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Docket No.: PF-0527-1 DIV

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By:

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Terence P. Lo

**EXPEDITED PROCEDURE  
EXAMINING GROUP 1642**



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Re Application of: Lal et al.

Title: PROSTATE GROWTH-ASSOCIATED MEMBRANE PROTEINS

Serial No.: 09/397,558

Filing Date: September 16, 1999

Examiner: Harris, A.

Group Art Unit: 1642

Commissioner for Patents  
Washington, D.C. 20231

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**BRIEF ON APPEAL**

Sir:

Further to the Notice of Appeal filed May 9, 2001, and received at the Patent Office on May 15, 2001, herewith are three copies of Appellants' Brief on Appeal. Appellants hereby request a one-month extension of time in order to file this Brief. Authorized fees include the statutory fee of \$110.00 for a one-month extension of time, as well as the \$310.00 fee for the filing of this Brief.

This is an appeal from the decision of the Examiner finally rejecting claims 1, 2, 21, 22, and 27-29 of the above-identified application.

**(1) REAL PARTY IN INTEREST**

The above-identified application is assigned of record to Incyte Pharmaceuticals, Inc. (now Incyte Genomics, Inc.), (Reel 9403, Frame 0092) who is the real party in interest herein.

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(2) RELATED APPEALS AND INTERFERENCES

Appellants, their legal representative and the assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the instant appeal.

(3) STATUS OF THE CLAIMS

Claims rejected:	Claims 1, 2, 21, 22, and 27-29
Claims allowed:	none
Claims canceled:	Claims 3-13 and 19-20
Claims withdrawn:	Claims 14-18, 23-26, and 30-32
Claims on Appeal:	Claims 1, 2, 21, 22, and 27-29 (A copy of the claims on appeal, as amended, can be found in the attached Appendix.)

(4) STATUS OF AMENDMENTS AFTER FINAL

The Amendment after Final Rejection under 37 C.F.R. § 1.116, mailed May 9, 2001, has been entered. See the Interview Summary of August 15, 2001.

(5) SUMMARY OF THE INVENTION

Appellants' invention is directed to polypeptides, human prostate growth-associated membrane proteins PGAMP-1 and PGAMP-2, comprising the amino acid sequences of SEQ ID NO:1 and SEQ ID NO:2, respectively (Specification, e.g., at page 13, line 33 to page 14, line 1; and page 14, lines 20-21). Appellants' invention also includes polypeptides comprising a naturally-occurring amino acid sequence having at least 90% amino acid sequence identity to either SEQ ID NO:1 or SEQ ID NO:2 (e.g., at page 15, lines 9-11), polypeptides comprising polypeptide fragments consisting of at least 15 contiguous amino acids of SEQ ID NO:1 (e.g., at page 7, lines 9-12), and compositions comprising polypeptides of SEQ ID NO:1 and SEQ ID NO:2 (e.g., at page 26, lines 17-19).

PGAMP-1 and PGAMP-2 have strong chemical and structural homology to rat heat-stable antigen CD4 (GenBank ID 1216498; SEQ ID NO:5), mouse apoptosis-associated tyrosine kinase (GenBank ID 2459993; SEQ ID NO:6), and human prostate specific antigen (GenBank ID

130989; SEQ ID NO:7) (Specification, e.g., at page 14, lines 6-7; page 14, lines 30-32; and Figures 1 and 2). In particular, PGAMP-1 and rat heat-stable antigen CD4 share 21% identity, PGAMP-2 and a fragment of mouse apoptosis-associated tyrosine kinase share 17% identity, and PGAMP-2 and human prostate specific antigen share 18% identity (e.g., at page 14, lines 7-8; and page 14, lines 32-33). In addition:

1. "PGAMP-1 is 141 amino acids in length and has one potential casein kinase II phosphorylation site at residue S35; one potential protein kinase C phosphorylation site at residue S15; one potential tyrosine kinase phosphorylation site at residue Y110; three potential transmembrane regions between about residues I44 to P67, I81 to W102, and P117 to Q135; and has chemical similarity with CD44 antigen precursor. . . Northern analysis shows the expression of this sequence in various libraries, at least 72% of which are immortalized or cancerous and at least 18% of which involve immune response. Of particular note is the expression of PGAMP in cancerous or hyperplastic prostate (48%) and breast (7%); and in brain and adrenal gland." (Specification at page 14, lines 1-13)
2. "PGAMP-2 is 410 amino acids in length and has a potential N-glycosylation site at residue N273; one potential cAMP- and cGMP-dependent protein kinase phosphorylation site at residue S355; one potential casein kinase II phosphorylation site at residue S274; seven potential protein kinase C phosphorylation sites at residues T118, S121, T131, S274, S311, S366, and S378; one potential tyrosine kinase phosphorylation site at residue Y21. In addition a hydropathy plot of PGAMP-2 predicts nine potential transmembrane regions between about residues L16 to Y31, P37 to V49, Q51 to Q73, V76 to L92, N101 to T118, F137 to F155, I165 to P182, R230 to W251, and T400 to V410; and a potential signal peptide sequence from M1 to S12. . . The three proteins [PGAMP-2, mouse apoptosis-associated tyrosine kinase, and human prostate specific antigen] also share six transmembrane regions and the potential signal peptide. In addition, PGAMP-2 and human PSA have rather similar isoelectric points, 8.7 and 7.5, respectively. . . Northern analysis shows the expression of this sequence in various libraries, at least 76% of which are immortalized or cancerous and at least 18% of which involve immune response. Of particular note is the expression of PGAMP-2 in cancerous or hyperplastic prostate (28%) and breast (10%); and in uterus, ovary, and colon." (Specification at page 14, lines 21-33 and page 15, lines 1-8)

The polypeptides of the present invention are useful, for example, for toxicology testing, drug discovery, and disease diagnosis.

#### (6) THE REJECTIONS

Claims 1, 2, 21, 22, and 27-29 stand rejected under 35 U.S.C. § 101 based on the allegation that the claimed invention is not supported by either a specific and substantial asserted

utility or a well established utility (Office Action, Feb. 9, 2001, page 5, section 7). Claims 1, 2, 21, 22, and 27-29 also stand rejected under 35 U.S.C. § 112, first paragraph, based on the allegation that the specification does not enable one of skill in the art to make and/or use the claimed invention (Office Action, Feb. 9, 2001, page 3, section 5; and page 6, section 7). These rejections allege in particular that:

- "Mere expression of PGAMP-1 or PGAMP-2 molecules in tissues does not mean effective treatment of disorders. There continues to be no objective evidence of record to show that these molecules can be used in the identification of cancerous tissue and as selective markers just for prostate cancer. Asserting that the claimed invention has strong chemical and structural homology to three other[s] does not support the molecules' utility nor applicability as therapeutic or pharmaceutical agents" (Addendum to Advisory Action, July 18, 2001, paragraph A).

Claim 2 stands rejected under 35 U.S.C. § 112, first paragraph, based on the allegation that the specification does not reasonably provide enablement commensurate with the scope of the claimed invention (Addendum to Advisory Action, July 18, 2001, paragraph B). This rejection alleges in particular that:

- "Applicants have yet to identify the amino acid residues that could [be] mutated, deleted or substituted that could yield polypeptides that would retain the structure and function in the same manner as Applicants have alleged" (Addendum to Advisory Action, July 18, 2001, paragraph B).

Claims 1, 2, 21, 22, and 27-29 stand rejected under 35 U.S.C. § 112, first paragraph, based on the allegation that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention (Office Action, February 9, 2001, page 3, section 5). This rejection alleges in particular that:

- "One skilled in the art cannot rely on Applicants expressed opinions and the percentages recited in the specification and in Applicants' arguments of Paper 12, pages 6 and 7 as definitive proof that SEQ ID Nos:1 and 2 as compounds that possibly can be used as diagnostic tools, therapeutics agents or as pharmaceutical agents. Thus, undue

experimentation would be required to use the instantly claimed polypeptides." (Office Action, February 9, 2001, page 4).

(7) ISSUES

1. Whether claims 1, 2, 21, 22, and 27-29 meet the utility requirement of 35 U.S.C. § 101.
2. Whether claims 1, 2, 21, 22, and 27-29 meet the enablement requirement of 35 U.S.C. § 112, first paragraph, *i.e.*, would the Specification enable one of ordinary skill in the art to make and use the claimed sequences, *e.g.*, in toxicology testing, drug development, and the diagnosis of disease.
3. Whether claim 2 meets the enablement requirement of 35 U.S.C. § 112, first paragraph, with respect to "variant" polypeptide sequences.
4. Whether claims 1, 2, 21, 22, and 27-29 meet the enablement requirement of 35 U.S.C. § 112, first paragraph, with respect to whether undue experimentation would be required to use the claimed invention.

(8) GROUPING OF THE CLAIMS

**As to Issue 1**

All of the claims on appeal are grouped together.

**As to Issue 2**

All of the claims on appeal are grouped together.

**As to Issue 3**

Claim 2 is grouped by itself.

**As to Issue 4**

All of the claims on appeal are grouped together.

(9) APPELLANTS' ARGUMENTS

**Issue 1 – Whether the claims meet the utility requirement of 35 U.S.C. § 101**

**The rejection of claims 1, 2, 21, 22, and 27-29 is improper, as the claims have a patentable utility as set forth in the instant specification, and/or a utility well-known to one of ordinary skill in the art.**

The invention at issue includes polypeptide sequences expressed in human tissues, including reproductive, neuronal, and gastrointestinal tissues, and tissues associated with cancer and the immune response (Specification, e.g., at page 25, lines 15-16; and page 25, lines 20-21). As such, the claimed inventions have numerous practical, beneficial uses in toxicology testing, drug development, and the diagnosis of disease, none of which necessarily require detailed knowledge of how the polypeptides work. As a result of the benefits of these uses, the claimed inventions already enjoy significant commercial success.

Any of these uses meets the utility requirements of 35 U.S.C. § 101 and, derivatively, § 112, first paragraph. Under these sections of the Patent Act, the patent applicant need only show that the claimed invention is "practically useful," *Anderson v. Natta*, 480 F.2d 1392, 1397, 178 USPQ 458 (CCPA 1973) and confers a "specific benefit" on the public. *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689 (1966). As discussed in a recent Court of Appeals for the Federal Circuit case, this threshold is not high:

An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See *Brenner v. Manson*, 383 U.S. 519, 534 [148 USPQ 689] (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 [24 USPQ2d 1401] (Fed. Cir. 1992) ("to violate Section 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end").

*Juicy Whip Inc. v. Orange Bang Inc.*, 51 USPQ2d 1700 (Fed. Cir. 1999). In *Stiftung v. Renishaw PLC*, 945 F.2d 1173, 1180, 20 USPQ2d 1094 (Fed. Cir. 1991) the United States Court of Appeal for the Federal Circuit explained:

An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: "[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 USPQ 473, 480 (Fed. Cir. 1984).

If persons of ordinary skill in the art would understand that there is a "well-established" utility for the claimed invention, the threshold is met automatically and the applicant need not make any showing to demonstrate utility. Manual of Patent Examination Procedure at § 706.03(a). Only if there is no "well-established" utility for the claimed invention must the applicant demonstrate the practical benefits of the invention. *Id.*

Once the patent applicant identifies a specific utility, the claimed invention is presumed to possess it. *In re Cortright*, 165 F.3d 1353, 1357, 49 USPQ2d 1464; *In re Brana*, 51 F.3d 1560, 1566; 34 USPQ2d 1436 (Fed. Cir. 1995). In that case the Patent Office bears the burden to demonstrate that a person of ordinary skill in the art would reasonably doubt that the asserted utility could be achieved by the claimed invention. *Ids.* To do so, the PTO must provide evidence or sound scientific reasoning. *See In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). If and only if the Patent Office makes such a showing, the burden shifts to the applicant to provide rebuttal evidence that would convince the person of ordinary skill that there is sufficient proof of utility. *Brana*, 51 F.3d at 1566. The applicant need only prove a "substantial likelihood" of utility; certainty is not required. *Brenner*, 383 U.S. at 532.

The rejections fail to demonstrate either that the Applicants' assertions of utility are legally insufficient or that a person of ordinary skill in the art would reasonably doubt that they could be achieved. For these reasons alone the rejections should be withdrawn.

There is, however, an additional, independent reason to overturn the rejections: to the extent the rejections are based on Revised Interim Utility Examination Guidelines (64 FR 71427, December 21, 1999), the final Utility Examination Guidelines (66 FR 1092, January 5, 2001), and/or the Revised Interim Utility Guidelines Training Materials (USPTO Website [www.uspto.gov](http://www.uspto.gov), March 1, 2000), the Guidelines and Training Materials are themselves inconsistent with the law. These inconsistencies are discussed separately below.

- I. **Use in drug discovery as screening tools for identifying agonists and antagonists; as diagnostics for cancer and reproductive and immunological disorders; as controls for monitoring expression of polypeptides in the monitoring of disease progression; as well as to develop and monitor the activities of therapeutic agents, and in particular, for the well-known specific use in toxicological studies for new drug development, are sufficient utilities under 35 U.S.C. §§ 101 and 112, first paragraph.**

The claimed invention meets all of the necessary requirements for establishing a credible utility under the Patent Law: There is a "well-established" use for the claimed invention, there are specific practical and beneficial uses for the invention, and those uses are substantial. Objective evidence, not considered by the Patent Office, further corroborates the credibility of the asserted utilities.

**A. The use of human polynucleotides and their encoded polypeptides as tools for toxicology testing, drug discovery, and the diagnosis of disease, is "well-established."**

In recent years, scientists have developed important techniques for toxicology testing, drug development, and disease diagnosis. Many of these techniques rely on expression profiling, in which the expression of numerous genes is compared in two or more samples. Genes or gene fragments known to be expressed are tools essential to any technology that uses expression profiling. Likewise, proteome expression profiling techniques have been developed in which the expression of numerous polypeptides is compared in two or more samples. Polypeptides known to be expressed, such as the invention at issue, are tools essential to any technology that uses proteome expression profiling. See, *e.g.*, Sandra Steiner and N. Leigh Anderson, Expression profiling in toxicology -- potentials and limitations, Toxicology Letters 112-113:467 (2000).

The technologies made possible by expression profiling and the DNA and polypeptide tools upon which they rely are now well-established. The technical literature recognizes not only the prevalence of these technologies, but also their unprecedented advantages in drug development, testing and safety assessment. One of these techniques is toxicology testing, used in both drug development and safety assessment. Toxicology testing is now standard practice in the pharmaceutical industry. See, *e.g.*, John C. Rockett, et al., Differential gene expression in drug metabolism and toxicology: practicalities, problems, and potential, Xenobiotica 29(7):655, 656 (1999):

Knowledge of toxin-dependent regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs.

To the same effect are several other scientific publications, including Emile F. Nuwaysir, et al., Microarrays and Toxicology: The Advent of Toxicogenomics, Molecular Genesis 24:153 (1999);

Sandra Steiner and N. Leigh Anderson, *supra*.

Nucleic acids useful for measuring the expression of whole classes of genes are routinely incorporated for use in toxicology testing. Nuwaysir et al. describes, for example, a Human ToxChip comprising 2089 human clones, which were selected

... for their well-documented involvement in basic cellular processes as well as their responses to different types of toxic insult. Included on this list are DNA replication and repair genes, apoptosis genes, and genes responsive to PAHs and dioxin-like compounds, peroxisome proliferators, estrogenic compounds, and oxidant stress. Some of the other categories of genes include transcription factors, oncogenes, tumor suppressor genes, cyclins, kinases, phosphatases, cell adhesion and motility genes, and homeobox genes. Also included in this group are 84 housekeeping genes, whose hybridization intensity is averaged and used for signal normalization of the other genes on the chip.

See also Table 1 of Nuwaysir et al. (listing additional classes of genes deemed to be of special interest in making a human toxicology microarray).

The more genes that are available for use in toxicology testing, the more powerful the technique. "Arrays are at their most powerful when they contain the entire genome of the species they are being used to study." John C. Rockett and David J. Dix, Application of DNA Arrays to Toxicology, Environ. Health Perspec. 107:681, No. 8 (1999). Control genes are carefully selected for their stability across a large set of array experiments in order to best study the effect of toxicological compounds. See attached email from the primary investigator, Dr. Cynthia Afshari to an Incyte employee, dated July 3, 2000, as well as the original message to which she was responding. Thus, there is no expressed gene which is irrelevant to screening for toxicological effects, and all expressed genes have a utility for toxicological screening. This is true for both polynucleotides and the polypeptides encoded by them.

There are numerous additional uses for the information made possible by expression profiling. Expression profiling is used to identify drug targets and characterize disease. See Rockett et al., *supra*. It also is used in tissue profiling, developmental biology, disease staging, etc. There is simply no doubt that the sequences of expressed human genes all have practical, substantial and credible real-world utilities, at the very least for expression profiling.

Expression profiling technology is also used to identify drug targets and analyze disease at the molecular level, thus accelerating the drug development process. For example, expression profiling is useful for the elucidation of biochemical pathways, each pathway comprising a

multitude of component polypeptides and thus providing a pool of potential drug targets. In this manner, expression profiling leads to the optimization of drug target identification and a comprehensive understanding of disease etiology and progression.

There is simply no doubt that the sequences of expressed human polynucleotides and polypeptides all have practical, substantial and credible real-world utilities, at the very least for biochemical pathway elucidation, drug target identification, and assessment of toxicity and treatment efficacy in the drug development process. Sandra Steiner and N. Leigh Anderson, *supra*, have elaborated on this topic as follows:

The rapid progress in genomics and proteomics technologies creates a unique opportunity to dramatically improve the predictive power of safety assessment and to accelerate the drug development process. Application of gene and protein expression profiling promises to improve lead selection, resulting in the development of drug candidates with higher efficacy and lower toxicity. The identification of biologically relevant surrogate markers correlated with treatment efficacy and safety bears a great potential to optimize the monitoring of pre-clinical and clinical trials.

In fact, the potential benefit to the public, in terms of lives saved and reduced health care costs, are enormous. Recent developments provide evidence that the benefits of this information are already beginning to manifest themselves. Examples include the following:

- In 1999, CV Therapeutics, an Incyte collaborator, was able to use Incyte gene expression technology, information about the structure of a known transporter gene, and chromosomal mapping location, to identify the key gene associated with Tangiers disease. This discovery took place over a matter of only a few weeks, due to the power of these new genomics technologies. The discovery received an award from the American Heart Association as one of the top 10 discoveries associated with heart disease research in 1999.
- In an April 9, 2000, article published by the Bloomberg news service, an Incyte customer stated that it had reduced the time associated with target discovery and validation from 36 months to 18 months, through use of Incyte's genomic information database. Other Incyte customers have privately reported similar experiences. The implications of this significant saving of time and expense for the number of drugs that may be developed and their cost are obvious.
- In a February 10, 2000, article in the *Wall Street Journal*, one Incyte customer stated that over 50 percent of the drug targets in its current pipeline were derived from the Incyte database. Other Incyte customers have privately reported similar experiences. By doubling the number of targets available to pharmaceutical researchers, Incyte genomic information has demonstrably accelerated the development of new drugs.

Because the Patent Examiner failed to address or consider the "well-established" utilities for the claimed invention in toxicology testing, drug development, and the diagnosis of disease, the Examiner's rejections should be withdrawn regardless of their merit.

**B. The use of the claimed polypeptides for toxicology testing, drug discovery, and disease diagnosis are practical uses that confer "specific benefits" to the public.**

Even if, *arguendo*, toxicology testing, drug development and disease diagnosis (through expression profiling) are not well-established utilities (which expressly is not conceded), the claimed invention nonetheless has specific utility by virtue of its use in each of these techniques. There is no dispute that the claimed invention is in fact a useful tool in each of these techniques. That is sufficient to establish utility for both the polypeptides and the polynucleotides encoding them.

Nevertheless, the claimed invention is rejected on the grounds that it does not have a "specific utility" absent a detailed description of the actual function of the claimed protein or identification of a "specific" disease it can be used to diagnose or treat. Apparently relying on the Training Materials, the rejection is made based on a scientifically incorrect and legally unsupportable assertion that identification of the family or families of proteins, without more, does not satisfy the utility requirement. None of these grounds is consistent with the law.

**1. A patent applicant can specify a utility without any knowledge as to how or why the invention has that utility.**

It is settled law that how or why any invention works is irrelevant to determining utility under 35 U.S.C. § 101: "[I]t is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works." *In re Cortright*, 165 F.3d, at 1359 (quoting *Newman v. Quigg*, 877 F.2d 1575, 1581, 11 USPQ2d 1340 (Fed. Cir. 1989)). *See also Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1570, 219 USPQ 1137 (Fed. Cir. 1983) ("[I]t is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests."). It follows that the patent applicant need not set forth the particular functionality of the claimed invention to satisfy the utility requirement.

Practical, beneficial use, not functionality, is at the core of the utility requirement. *Supra* (introduction to § I). So long as the practical benefits are apparent from the invention without speculation, the requirement is satisfied. *Standard Oil Co. v. Montedison*, 664 F.2d 356, 374,

212 USPQ 327 (3d Cir. 1981); *see also Brana*, 51 F.3d at 1565. To state that a biological molecule might be useful to treat some unspecified disease is not, therefore a specific utility. *In re Kirk*, 376 F.2d 936, 945, 153 USPQ 48 (C.C.P.A. 1967). The molecule might be effective, and it might not.

However, unlike the synthetic molecules of *Kirk*, the claimed invention is **known** to be useful. It is not just a random sequence of speculative use. Because it is expressed in humans, a person of ordinary skill in the art would know how to use the claimed polypeptide sequences -- without any guesswork -- in toxicology testing, drug development, and disease diagnosis regardless of how the protein actually functions. The claimed invention could be used, for example, in a toxicology test to determine whether a drug or toxin causes any change in the expression of molecules involved in cancer, or reproductive or immunological disorders. Similarly, the claimed invention could be used to determine whether a specific medical condition, such as prostate cancer, affects the expression of PGAMP proteins, and, perhaps in conjunction with other information, serve as a marker for or to assess the stage of a particular disease or condition.

In fact, the claimed invention could be used in toxicology testing and diagnosis without **any** knowledge (although this is not the case here) of the precise function of the protein: it could serve, for example, as a marker of a toxic response, or, alternatively, if levels of the claimed polypeptide remain unchanged during a toxic response, as a control in toxicology testing. Diagnosis of disease (or fingerprinting using expression profiles) can be achieved using arrays of numerous identifiable, expressed DNA sequences, or by two-dimensional gel analysis of the expressed proteins themselves, notwithstanding lack of any knowledge of the function of the proteins.

The claimed polypeptides can be used in protein expression analysis techniques such as 2-D PAGE gels and western blots. Using the claimed invention with these techniques, persons of ordinary skill in the art can better assess, for example, the potential toxic affect of a drug candidate. The Patent Examiner does not dispute that the claimed polypeptide can be used in 2-D PAGE gels and western blots to perform drug toxicity testing. Instead, the Patent Examiner contends that the claimed polypeptide cannot be useful without precise knowledge of its function. But the law never has required knowledge of biological function to prove utility. It is

the claimed invention's uses, not its functions, that are the subject of a proper analysis under the utility requirement.

**2. A patent applicant may specify a utility that applies to a broad class of inventions.**

The fact that the claimed invention is a member of a broad class (such as DNA sequences expressed in humans, or the human proteins they encode) that includes sequences other than those claimed that also have utilities in toxicology testing, drug discovery, disease diagnosis, etc. does not negate utility. Practical utilities can be directed to classes of inventions, irrespective of function, so long as a person of ordinary skill in the art would understand how to achieve a practical benefit from knowledge of the class. *Montedison*, 664 F.2d at 374-75. The law has long assumed that inventions that achieve a practical use also achieved by other inventions satisfy the utility requirement. For example, many materials conduct electricity. Likewise, many different plastics can be used to form useful films. *Montedison*, 664 F.2d at 374-75; *Natta*, 480 F.2d at 1397. This is a general utility (practical films) that applies to a broad class of inventions (plastics) which satisfies the utility requirement of 35 U.S.C. § 101.

Not all broad classes of inventions are, by themselves, sufficient to inform a person of ordinary skill in the art of the practical utility for a member of the class. Some classes may indeed convey too little information to a person of ordinary skill in the art. These may include classes of inventions that include both useful and nonuseful members. See *In re Ziegler*, 992 F.2d 1197, 1201, 26 USPQ2d 1600 (Fed. Cir. 1993). In some of these cases, further experimentation would be required to determine whether or not a member of the class actually has a practical use. *Brenner*, 383 U.S. at 534-35.

The broad class of steroids identified in *Kirk* is just such a class. It includes natural steroids (concededly useful) and man-made steroids, some of which are useful and some of which are not. Indeed, only a small fraction of the members of this broad class of invention may be useful. Without additional information or further experimentation, a person of ordinary skill in the art would not know whether a member of the class falls into the useful category or not. This could also be the case for the broad class of "plastic-like" polypropylenes in *Ziegler*, which includes many -- perhaps predominately -- useless members.

The PTO routinely issues patents whose utility is based solely on the claimed inventions'

membership in a class of useful things. The PTO presumably would issue a patent on a novel and nonobvious fishing rod notwithstanding the lack of any disclosure of the particular fish it might be used to catch. The standard being promulgated in the Guidelines and in particular as exemplified in the Training Materials, and being applied in the present rejection, would appear to warrant a rejection, however, on the grounds that the use of the fishing rod is applicable to the general class of devices used to catch fish.

The PTO must apply the same standard to the biotechnological arts that it applies to fields such as plastics and fishing equipment. *In re Gazave*, 379 F.2d 973, 977-78, 154 USPQ 92 (CCPA 1967) quoting *In re Chilowsky*, 299 F.2d 457, 461, 108 USPQ 321 (CCPA 1956) ("[T]he same principles should apply in determining operativeness and sufficiency of disclosure in applications relating to nuclear fission art as in other cases."); see also *In re Alappat*, 33 F.3d 1526, 1566, 31 USPQ2d 1545 (Fed. Cir. 1994) (Archer, C.J., concurring in part and dissenting in part) ("Discoveries and inventions in the field of digital electronics are analyzed according to the aforementioned principles [concerning patentable subject matter] as any other subject matter."). Indeed, there are numerous classes of inventions in the biotechnological arts that satisfy the utility requirement.

Take, for example, the class of interleukins expressed in human cells of the immune system. Unlike the classes of steroids or plastic-like polypropylenes in *Kirk* and *Ziegler*, all of the members of this class have practical uses well beyond "throwaway" uses. All of them cause some physiological response (in cells of the immune system). All of the genes encoding them can be used for toxicology testing to generate information useful in activities such as drug development, even in cases where little is known as to how a particular interleukin works. No additional experimentation would be required, therefore, to determine whether an interleukin has a practical use. It is well-known to persons of ordinary skill in the art that there is no such thing as a useless interleukin.

Because all of the interleukins, as a class, convey practical benefit (much like the class of DNA ligases identified in the Training Materials), there is no need to provide additional information about them. A person of ordinary skill in the art need not guess whether any given interleukin conveys a practical benefit or how that particular interleukin works.

Another example of a class that by itself conveys practical benefits is the G protein-

coupled receptors ("GPCRs"). GPCRs are well-known as intracellular signaling mediators with diverse functions critical to complex organisms. They perform these functions by binding to and interacting with specific ligands. They are targets of many current drug treatments, including anti-depressants, anti-histamines, blood pressure regulators, and opiates.

Newly-identified GPCRs are used intensively in the real-world, even in cases where neither the specific ligand that binds to the GPCR or the precise biological function of the GPCR is known. Newly identified GPCRs are used, for example, as toxicity controls for drug candidates known to bind other GPCRs. Because a person of ordinary skill in the art would know how to use any GPCR to achieve a practical benefit, even without any detailed or particular knowledge as to how it works, GPCRs as a class meet the utility requirement.

In fact, all isolated and purified naturally-occurring polynucleotide and polypeptide sequences which are expressible (i.e., which are not pseudogenes that are never expressed during any natural biological process) can be and **are** used in a real-world context as tools for toxicological testing, e.g., for drug discovery purposes. This utility applies to all sequences actually expressed, yet in each case, the utility of the sequence is quite specific, e.g., monitoring the expression of any particular polynucleotide or polypeptide sequence is a utility specific to that particular sequence.

Prostate growth-associated membrane proteins, like interleukins, GPCRs and fishing rods, is a class that by itself conveys practical benefits. Unlike steroids and "plastic-like" polypropylenes, all of the claimed prostate growth-associated membrane proteins are expressed by humans, and all of them can be used as tools for toxicology testing. The claimed invention could be used, for example to determine whether a drug candidate affects the expression of prostate growth-associated membrane proteins in humans, how it does so, and to what extent. Just as there are no useless interleukins and GPCRs, there are no useless prostate growth-associated membrane proteins of the claimed invention. As these are practical, real-world uses, the application need not describe particular functionality or medical applications that would only supplement the utilities known to exist already.

- C. Because the use of prostate growth-associated membrane proteins in toxicology testing, drug discovery, and disease diagnosis are practical uses beyond mere study of the invention itself, the claimed invention has substantial utility.**

In addition to conferring a specific benefit on the public, the benefit must also be "substantial." *Brenner*, 383 U.S. at 534. A "substantial" utility is a practical, "real-world" utility. *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881 (CCPA 1980).

The claimed invention's use as a tool for toxicology testing is just such a practical, real-world use. Although the rejection is not expressly based on lack of practical utility, and/or ignores this basis for utility, as stated it is tantamount to a rejection based on the sequence being only a research tool, on the ground that the use of an invention as a tool for research is not a "substantial" use. Because the PTO's rejection in this light assumes a substantial overstatement of the law, it must be withdrawn.

There is no authority for the proposition that use as a tool for research is not a substantial utility. In fact, the PTO issues patents for inventions whose only use is to facilitate research, such as DNA ligases. These are acknowledged by the PTO's Training Materials themselves to be useful.

Only a limited subset of research uses are not "substantial" utilities: those in which the only known use for the claimed invention is to be an **object** of further study, thus merely inviting further research. This follows from *Brenner*, in which the U.S. Supreme Court held that a process for making a compound does not confer a substantial benefit where the only known use of the compound was to be the object of further research to determine its use. *Id.* at 535. Similarly, in *Kirk*, the CCPA held that a compound would not confer substantial benefit on the public merely because it might be used to synthesize some other, unknown compound that would confer substantial benefit. *Kirk*, 376 F.2d at 940, 945 ("What appellants are really saying to those in the art is take these steroids, experiment, and find what use they do have as medicines."). Nowhere do those cases state or imply, however, that a material cannot be patentable if it has some other beneficial use in research.

As used in toxicology testing, drug discovery, and disease diagnosis, the claimed invention has a beneficial use in research other than studying the claimed invention. It is a tool, rather than an object, of research. The claimed invention has numerous other uses as a research tool, each of which alone is a "substantial utility". These include diagnostic assays (pages 33-36), drug screening (page 38), etc.

**D. Objective evidence corroborates the utilities of the claimed invention.**

There is in fact no restriction on the kinds of evidence a Patent Examiner may consider in determining whether a "real-world" utility exists. Indeed, "real-world" evidence, such as evidence showing actual use or commercial success of the invention, can demonstrate conclusive proof of utility. *Raytheon v. Roper*, 220 USPQ2d 592 (Fed. Cir. 1983); *Nestle v. Eugene*, 55 F.2d 854, 856, 12 USPQ 335 (6th Cir. 1932). Indeed, proof that the invention is made, used or sold by any person or entity other than the patentee is conclusive proof of utility. *United States Steel Corp. v. Phillips Petroleum Co.*, 865 F.2d 1247, 1252, 9 USPQ2d 1461 (Fed. Cir. 1989).

Over the past several years, a vibrant market has developed for databases containing all expressed genes (along with the polypeptide translations of those genes), in particular genes having medical and pharmaceutical significance such as the instant sequence. (Note that the value in these databases is enhanced by their completeness, but each sequence in them is independently valuable.) The databases sold by Applicants' assignee, Incyte, include exactly the kinds of information made possible by the claimed invention, such as tissue and disease associations. Incyte sells its database containing the claimed sequence and millions of other sequences throughout the scientific community, including to pharmaceutical companies who use the information to develop new pharmaceuticals.

**II. The Patent Examiner failed to demonstrate that a person of ordinary skill in the art would reasonably doubt the utility of the claimed invention.**

In addition to alleging a "specific" use for the claimed subject matter, a patent applicant must present proof that the claimed subject matter is in fact useful. *Brana*, 51 F.3d at 1565-66. The applicant need only prove a "substantial likelihood" of utility; certainty is not required. *Brenner*, 383 U.S. at 532.

The amount of evidence required to prove utility depends on the facts of each particular case. *In re Jolles*, 628 F.2d 1322, 1326, 206 USPQ 885 (CCPA 1980). "The character and amount of evidence may vary, depending on whether the alleged utility appears to accord with or to contravene established scientific principles and beliefs." *Id.* Unless there is proof of "total incapacity," or there is a "complete absence of data" to support the applicant's assertion of utility, the utility requirement is met. *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571, 24 USPQ2d 1401 (Fed. Cir. 1992); *Envirotech*, 730 F.2d at 762.

A patent applicant's assertion of utility in the disclosure is presumed to be true and correct. *In re Cortright*, 165 F.3d at 1356; *Brana*, 51 F.3d at 1566. If such an assertion is made, the Patent Office bears the burden in the first instance to demonstrate that a person of ordinary skill in the art would reasonably doubt that the asserted utility could be achieved. *Ids.* To do so, the PTO must provide evidence or sound scientific reasoning. *See Langer*, 503 F.2d at 1391-92. If and only if the Patent Office makes such a showing, the burden shifts to the applicant to provide rebuttal evidence that would convince the person of ordinary skill that there is sufficient proof of utility. *Brana*, 51 F.3d at 1566. The Revised and final Utility Guidelines are in agreement with this procedure. *See Revised and final Guidelines at ¶¶ 3-4.*

The issue of proof often arises in the chemical and biotechnological arts when the patentee asserts a utility for a claimed chemical compound based on its homology or similarity to another compound having a known, established utility. In such cases, the applicant can demonstrate "substantial likelihood" of utility by demonstrating a "reasonable correlation" between the utility -- not the function -- of the known compound and the compound being claimed. *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1565, 39 USPQ2d 1895 (Fed. Cir. 1996). Accordingly, under *Brana*, the Patent Office must accept the asserted utility unless it can show that a person of ordinary skill in the art would reasonably doubt that a "reasonable correlation" exists.

In the instant case, the Patent Examiner has not addressed the asserted utilities of the claimed polypeptides as research tools, for example, in toxicology testing, diagnostic assays, and drug screening. As such, the Patent Office has not met its burden to demonstrate that a person of ordinary skill in the art would reasonably doubt that the asserted utilities could be achieved. Therefore, the rejection of the claimed invention for lack of utility under 35 U.S.C. § 101 should be reversed.

### **III. By requiring the Patent Applicant to assert a particular or unique utility, the Patent Examination Utility Guidelines and Training Materials applied by the Patent Examiner misstate the Law.**

The Training Materials, which direct the Examiners regarding how to apply the Utility Guidelines, address the issue of specificity with reference to two kinds of asserted utilities: "specific" utilities which meet the statutory requirements, and "general" utilities which do not. The Training Materials define a "specific utility" as follows:

A [specific utility] is *specific* to the subject matter claimed. This contrasts to *general* utility that would be applicable to the broad class of invention. For example, a claim to a polynucleotide whose use is disclosed simply as "gene probe" or "chromosome marker" would not be considered to be specific in the absence of a disclosure of a specific DNA target. Similarly, a general statement of diagnostic utility, such as diagnosing an unspecified disease, would ordinarily be insufficient absent a disclosure of what condition can be diagnosed.

The Training Materials distinguish between "specific" and "general" utilities by assessing whether the asserted utility is sufficiently "particular," *i.e.*, unique (Training Materials at p.52) as compared to the "broad class of invention." (In this regard, the Training Materials appear to parallel the view set forth in Stephen G. Kunin, Written Description Guidelines and Utility Guidelines, 82 J.P.T.O.S. 77, 97 (Feb. 2000) ("With regard to the issue of specific utility the question to ask is whether or not a utility set forth in the specification is *particular* to the claimed invention.")).

Such "unique" or "particular" utilities never have been required by the law. To meet the utility requirement, the invention need only be "practically useful," *Natta*, 480 F.2d 1 at 1397, and confer a "specific benefit" on the public. *Brenner*, 383 U.S. at 534. Thus incredible, "throw-away" utilities, such as trying to "patent a transgenic mouse by saying it makes great snake food" do not meet this standard. Karen Hall, Genomic Warfare, *The American Lawyer* 68 (June 2000) (quoting John Doll, Chief of the Biotech Section of USPTO).

This does not preclude, however, a general utility, contrary to the statement in the Training Materials where "specific utility" is defined (page 5). Practical real-world uses are not limited to uses that are unique to an invention. The law requires that the practical utility be "definite," not particular. *Montedison*, 664 F.2d at 375. Appellant is not aware of any court that has rejected an assertion of utility on the grounds that it is not "particular" or "unique" to the specific invention. Where courts have found utility to be too "general," it has been in those cases in which the asserted utility in the patent disclosure was not a practical use that conferred a specific benefit. That is, a person of ordinary skill in the art would have been left to guess as to how to benefit at all from the invention. In *Kirk*, for example, the CCPA held the assertion that a man-made steroid had "useful biological activity" was insufficient where there was no information in the specification as to how that biological activity could be practically used. *Kirk*, 376 F.2d at 941.

The fact that an invention can have a particular use does not provide a basis for requiring a particular use. *See Brana, supra* (disclosure describing a claimed antitumor compound as being homologous to an antitumor compound having activity against a "particular" type of cancer was determined to satisfy the specificity requirement). "Particularity" is not and never has been the *sine qua non* of utility; it is, at most, one of many factors to be considered.

As described *supra*, broad classes of inventions can satisfy the utility requirement so long as a person of ordinary skill in the art would understand how to achieve a practical benefit from knowledge of the class. Only classes that encompass a significant portion of nonuseful members would fail to meet the utility requirement. *Supra* § I.B.2 (*Montedison*, 664 F.2d at 374-75).

The Training Materials fail to distinguish between broad classes that convey information of practical utility and those that do not, lumping all of them into the latter, unpatentable category of "general" utilities. As a result, the Training Materials paint with too broad a brush. Rigorously applied, they would render unpatentable whole categories of inventions heretofore considered to be patentable, and that have indisputably benefitted the public, including the claimed invention. *See supra* § I.B. Thus the Training Materials cannot be applied consistently with the law.

**Issue 2 – Whether the claims meet the enablement requirement of 35 U.S.C. § 112, first paragraph, with respect to the "utility" issue**

**To the extent the rejection of the patented invention under 35 U.S.C. § 112, first paragraph, is based on the improper rejection for lack of utility under 35 U.S.C. § 101, it must be reversed.**

The rejection set forth in the Office Action is based on the assertions discussed above, i.e., that the claimed invention lacks patentable utility. To the extent that the rejection under § 112, first paragraph, is based on the improper allegation of lack of patentable utility under § 101, it fails for the same reasons.

**Issue 3 – Whether claim 2 meets the enablement requirement of 35 U.S.C. § 112, first paragraph, with respect to "variant" polypeptide sequences**

Claim 2 recites polypeptides comprising naturally-occurring amino acid sequences having at least 90% amino acid sequence identity to either SEQ ID NO:1 or SEQ ID NO:2. This claim stands rejected under 35 U.S.C. § 112, first paragraph, based on the allegation that the specification does not reasonably provide enablement commensurate with the scope of the invention recited in this claim (Addendum to Advisory Action, July 18, 2001, paragraph B). The Patent Examiner stated that "Applicants are not entitled to all polypeptide variants that have at least 90% sequence identity" (Office Action, February 9, 2001, page 5, first paragraph), and that "Applicants have yet to identify the amino acid residues that could [be] mutated, deleted or substituted that could yield polypeptides that would retain the structure and function in the same manner as Applicants have alleged" (Addendum to Advisory Action, July 18, 2001, paragraph B).

Claim 2 does not recite **all** polypeptide variants that have at least 90% sequence identity to either SEQ ID NO:1 or SEQ ID NO:2. Rather, claim 2 recites only **naturally-occurring** amino acid sequences that have at least 90% sequence identity to either SEQ ID NO:1 or SEQ ID NO:2. One of ordinary skill in the art would know how to obtain naturally-occurring amino acid sequences, and would recognize whether such sequences had at least 90% sequence identity to either SEQ ID NO:1 or SEQ ID NO:2. Furthermore, one of ordinary skill in the art would know how to use the recited sequences as, for example, research tools in toxicology testing, diagnostic assays, or drug screening. Such uses do not require guidance as to which amino acid residues could be mutated, deleted, or substituted to yield polypeptides that would retain the structure and function of SEQ ID NO:1 or SEQ ID NO:2, because these uses depend only on the fact that the recited polypeptides are expressed in a living organism.

One of ordinary skill in the art would know how to make and use the polypeptide "variants" recited by claim 2. Therefore, reversal of this rejection of claim 2 under 35 U.S.C. § 112, first paragraph, based on the alleged lack of enablement of the recited polypeptide "variants," is requested.

**Issue 4 – Whether claims 1, 2, 21, 22, and 27-29 meet the enablement requirement of 35 U.S.C. § 112, first paragraph, with respect to whether undue experimentation would be required to use the claimed invention**

The requirements necessary to fulfill the enablement requirement of 35 U.S.C. 112, first

paragraph, are well established by case law.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 U.S.P.Q.2d 1217, 1223 (Fed. Cir. 1988).

Likewise, the Manual of Patent Examining Procedure (MPEP) states that the enablement requirement "has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation." (MPEP, 7<sup>th</sup> Edition, July 1998, Section 2164.01, page 2100-145.) The MPEP further states that, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation...[t]he test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue." (MPEP, 7<sup>th</sup> Edition, Section 2164.01(b), page 2100-147.)

The determination of enablement must be made based on consideration of the evidence as a whole and that, "the evidence provided by applicant need not be **conclusive** but merely **convincing** to one skilled in the art." (MPEP, 7<sup>th</sup> Edition, Section 2164.05, page 2100-150.) (Emphasis in original.)

Appellants respectfully submit that a proper *prima facie* case of lack of enablement has not been established. As was set forth by the Court of Appeals for the Federal Circuit in *In re Brana*, 34 U.S.P.Q.2d 1436 (C.A.F.C. 1995):

This court's predecessor has stated:

[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of Section 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971). From this it follows that the PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. *Id.* at 224, 169 USPQ at 370. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. See *In re Bundy*, 642 F.2d 430, 433, 209 USPQ 48, 51 (CCPA 1981).

The Examiner asserted in the Office Action dated February 9, 2001 (Paper Number 13, page 4), that "Applicants own submission admits to the fact that these polypeptides are not expressed solely in cancerous tissue so the applicability of the proteins is still at question. One skilled in the art can not rely on Applicants expressed opinions and the percentages recited in the specification and in Applicants' arguments of Paper 12, pages 6 and 7 as definitive proof that SEQ ID Nos:1 and 2 as compounds that possibly can be used as diagnostic tools, therapeutics agents or as pharmaceutical agents." However, to satisfy the enablement requirement, the burden does not fall on the Appellants to provide **definitive proof** that the polypeptides of SEQ ID NO:1 and SEQ ID NO:2 are useful as diagnostic tools, therapeutic agents, or pharmaceutical agents. The Appellants need only supply **convincing** evidence that the claimed invention is enabled by the specification.

The specification clearly discloses that at least 72% of the tissue libraries in which PGAMP-1 was detected were cancerous or immortalized, and at least 76% of the tissue libraries in which PGAMP-2 was detected were cancerous or immortalized (specification, e.g., at page 14, lines 10-12; and page 15, lines 4-6). One of skill in the art, upon reading this disclosure in the context of the patent application at the time of filing, would be **reasonably convinced** that the claimed polypeptides could be used as diagnostic tools, therapeutic agents, and/or pharmaceutical agents. The Examiner has provided no objective evidence or sound scientific reasoning to show that the claimed polypeptides, which "are not expressed solely in cancerous tissue," could not be used in the manner asserted by the Appellants. The Examiner has also not provided objective evidence or sound scientific reasoning to show that expression of the polypeptides solely in one type of tissue is a necessary requirement for using polypeptides, such as those claimed, in the manner asserted by the Appellants. Therefore, the Examiner has not met the burden to establish a *prima facie* case of lack of enablement. Therefore the decision of the Examiner rejecting the claims for lack of enablement should be reversed.

#### (10) CONCLUSION

Appellants respectfully submit that rejections for lack of utility based, *inter alia*, on an allegation of "lack of specificity" as set forth in the Office Action and as justified in the Revised Interim and final Utility Guidelines and Training Materials, are not supported in the law. Neither

are they scientifically correct, nor supported by any evidence or sound scientific reasoning. These rejections are alleged to be founded on facts in court cases such as *Brenner* and *Kirk*, yet those facts are clearly distinguishable from the facts of the instant application, and indeed most if not all nucleotide and protein sequence applications. Nevertheless, the PTO is attempting to mold the facts and holdings of these prior cases, "like a nose of wax,"<sup>1</sup> to target rejections of claims to polypeptide and polynucleotide sequences where biological activity information has not been proven by laboratory experimentation, and they have done so by ignoring perfectly acceptable utilities fully disclosed in the specification as well as well-established utilities known to those of skill in the art. As is disclosed in the specification, and even more clearly, as one of ordinary skill in the art would understand, the claimed invention has well-established, specific, substantial, and credible utilities. The "utility" rejections are, therefore, improper and should be reversed.

Moreover, to the extent the above rejections were based on the Revised Interim and final Examination Guidelines and Training Materials, those portions of the Guidelines and Training Materials that form the basis for the rejections should be determined to be inconsistent with the law.

The enablement rejections, with respect to the "variant" polypeptide sequences of claim 2, and the allegation that undue experimentation would be required to use the claimed invention, should also be reversed, based on at least the arguments presented above.

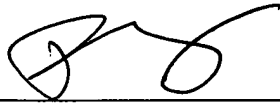
Due to the urgency of this matter, and its economic and public health implications, an expedited review of this appeal is earnestly solicited.

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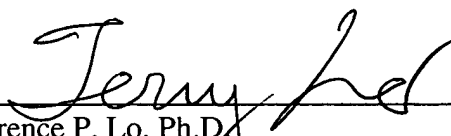
<sup>1</sup> "The concept of patentable subject matter under §101 is not 'like a nose of wax which may be turned and twisted in any direction \* \* \*.' *White v. Dunbar*, 119 U.S. 47, 51." (*Parker v. Flook*, 198 USPQ 193 (US SupCt 1978))

Respectfully submitted,  
INCYTE GENOMICS, INC.

Date: 8/15/01

  
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**APPENDIX**

Claims on appeal:

1. A substantially purified polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

2. An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

a) a naturally-occurring amino acid sequence having at least 90% amino acid sequence identity to SEQ ID NO:1, and

b) a naturally-occurring amino acid sequence having at least 90% amino acid sequence identity to SEQ ID NO:2.

21. A polypeptide of claim 1, having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

22. A composition comprising a polypeptide of claim 21 in conjunction with a suitable pharmaceutical carrier.

27. A composition comprising a polypeptide of claim 1 in conjunction with a suitable pharmaceutical carrier.

28. A substantially purified polypeptide comprising a fragment of the polypeptide of claim 1, wherein said fragment consists of at least 15 contiguous amino acids of SEQ ID NO:1, and wherein said fragment binds specifically with an anti-PGAMP-1 antibody.

29. A composition comprising the polypeptide of claim 28 in conjunction with a suitable pharmaceutical carrier.



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BEFORE THE OFFICE OF ENROLLMENT AND DISCIPLINE**

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**AUG 22 2001**


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**LIMITED RECOGNITION UNDER 37 CFR §10.9(b)**

Terrence Lo is hereby given limited recognition under 37 CFR §10.9(b) as an employee of Incyte Pharmaceuticals, Inc. to prepare and prosecute patent applications wherein Incyte Pharmaceuticals, Inc. is the assignee of record of the entire interest in the application filed in the USPTO. This limited recognition shall expire on the date appearing below, or when whichever of the following events first occurs prior to the date appearing below: (i) Terrence Lo ceases to lawfully reside in the United States; (ii) Terrence Lo's employment with Incyte Pharmaceuticals, Inc. ceases or is terminated; or (iii) Terrence Lo ceases to remain or reside in the United States on TN status.

This document constitutes proof of such recognition. The original of this document is on file in the Office of Enrollment and Discipline of the U.S. Patent and Trademark Office.

**Expires: November 18, 2001**

  
Harry I. Moatz  
Director of Enrollment and Discipline

cell's development or response and should help in the elucidation of specific and sensitive biomarkers representing, for example, different types of cancer or previous exposure to certain classes of chemicals that are enzyme inducers.

In drug metabolism, many of the xenobiotic-metabolizing enzymes (including the well-characterized isoforms of cytochrome P450) are inducible by drugs and chemicals in man (Pelkonen *et al.* 1998), predominantly involving transcriptional activation of not only the cognate cytochrome P450 genes, but additional cellular proteins which may be crucial to the phenomenon of induction. Accordingly, the development of methodology to identify and assess the full complement of genes that are either up- or down-regulated by inducers are crucial in the development of knowledge to understand the precise molecular mechanisms of enzyme induction and how this relates to drug action. Similarly, in the field of chemical-induced toxicity, it is now becoming increasingly obvious that most adverse reactions to drugs and chemicals are the result of multiple gene regulation, some of which are causal and some of which are casually-related to the toxicological phenomenon *per se*. This observation has led to an upsurge in interest in gene-profiling technologies which differentiate between the control and toxin-treated gene pools in target tissues and is, therefore, of value in rationalizing the molecular mechanisms of xenobiotic-induced toxicity. Knowledge of toxin-dependent gene regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. For example, if the gene profile in response to say a testicular toxin that has been well-characterized *in vivo* could be determined in the testis, then this profile would be representative of all new drug candidates which act via this specific molecular mechanism of toxicity, thereby providing a useful and coherent approach to the early detection of such toxicants. Whereas it would be informative to know the identity and functionality of all genes up/down regulated by such toxicants, this would appear a longer term goal, as the majority of human genes have not yet been sequenced, far less their functionality determined. However, the current use of gene profiling yields a *pattern* of gene changes for a xenobiotic of unknown toxicity which may be matched to that of well-characterized toxins, thus alerting the toxicologist to possible *in vivo* similarities between the unknown and the standard, thereby providing a platform for more extensive toxicological examination. Such approaches are beginning to gain momentum, in that several biotechnology companies are commercially producing 'gene chips' or 'gene arrays' that may be interrogated for toxicity assessment of xenobiotics. These chips consist of hundreds/thousands of genes, some of which are degenerate in the sense that not all of the genes are mechanistically-related to any one toxicological phenomenon. Whereas these chips are useful in broad-spectrum screening, they are maturing at a substantial rate, in that gene arrays are now becoming more specific, e.g. chips for the identification of changes in growth factor families that contribute to the aetiology and development of chemically-induced neoplasias.

Although documenting and explaining these genetic changes presents a formidable obstacle to understanding the different mechanisms of development and disease progression, the technology is now available to begin attempting this difficult challenge. Indeed, several 'differential expression analysis' methods have been developed which facilitate the identification of gene products that demonstrate

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## Differential gene expression in drug metabolism and toxicology: practicalities, problems and potential

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1. An important feature of the work of many molecular biologists is identifying which genes are switched on and off in a cell under different environmental conditions or subsequent to xenobiotic challenge. Such information has many uses, including the deciphering of molecular pathways and facilitating the development of new experimental and diagnostic procedures. However, the student of gene hunting should be forgiven for perhaps becoming confused by the mountain of information available as there appears to be almost as many methods of discovering differentially expressed genes as there are research groups using the technique.

2. The aim of this review was to clarify the main methods of differential gene expression analysis and the mechanistic principles underlying them. Also included is a discussion on some of the practical aspects of using this technique. Emphasis is placed on the so-called 'open' systems, which require no prior knowledge of the genes contained within the study model. Whilst these will eventually be replaced by 'closed' systems in the study of human, mouse and other commonly studied laboratory animals, they will remain a powerful tool for those examining less fashionable models.

3. The use of suppression-PCR subtractive hybridization is exemplified in the identification of up- and down-regulated genes in rat liver following exposure to phenobarbital, a well-known inducer of the drug metabolizing enzymes.

4. Differential gene display provides a coherent platform for building libraries and microchip arrays of 'gene fingerprints' characteristic of known enzyme inducers and xenobiotic toxicants, which may be interrogated subsequently for the identification and characterization of xenobiotics of unknown biological properties.

### Introduction

It is now apparent that the development of almost all cancers and many non-neoplastic diseases are accompanied by altered gene expression in the affected cells compared to their normal state (Hunter 1991, Wyntford-Thomas 1991, Vogelstein and Kinzler 1993, Semenza 1994, Cassidy 1995, Kleinjan and Van Hegningen 1998). Such changes also occur in response to external stimuli such as pathogenic micro-organisms (Rohn *et al.* 1996, Singh *et al.* 1997, Griffin and Krishna 1998, Lunney 1998) and xenobiotics (Sewall *et al.* 1995, Dogra *et al.* 1998, Ramana and Kohli 1998), as well as during the development of undifferentiated cells (Hecht 1998, Rudin and Thompson 1998, Schneider-Maunoury *et al.* 1998). The potential medical and therapeutic benefits of understanding the molecular changes which occur in any given cell in progressing from the normal to the 'altered' state are enormous. Such profiling essentially provides a 'fingerprint' of each step of a

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ation of specific and of cancer or previous ers. enzymes (including ucible by drugs and iving transcriptional at additional cellular on. Accordingly, the omplement of genes n the development of of enzyme induction of chemical-induced adverse reactions to n, some of which are ical phenomenon *per* rofiling technologies pools in target tissues nisms of xenobiotic- on in target tissues is n generated in the identification of toxic ess and contributing le, if the gene profile ized *in vivo* could be ative of all new drug of toxicity, thereby on of such toxicants. ctionality of all genes iger term goal, as the ss their functionality ds a *pattern* of gene atched to that of well- e *in vivo* similarities a platform for more beginning to gain mercially producing oxicity assessment of es, some of which are tically-related to any il in broad-spectrum gene arrays are now nges in growth factor chemically-induced

altered expression in cells of one population compared to another. These methods have been used to identify differential gene expression in many situations, including invading pathogenic microbes (Zhao *et al.* 1998), in cells responding to extracellular and intracellular microbial invasion (Duguid and Dinauer 1990, Ragno *et al.* 1997, Maldarelli *et al.* 1998), in chemically treated cells (Syed *et al.* 1997, Rockett *et al.* 1999), neoplastic cells (Liang *et al.* 1992, Chang and Terzaghi-Howe 1998), activated cells (Gurskaya *et al.* 1996, Wan *et al.* 1996), differentiated cells (Hara *et al.* 1991, Guimaraes *et al.* 1995a, b), and different cell types (Davis *et al.* 1984, Hedrick *et al.* 1984, Xhu *et al.* 1998). Although differential expression analysis technologies are applicable to a broad range of models, perhaps their most important advantage is that, in most cases, absolutely no prior knowledge of the specific genes which are up- or down-regulated is required.

The field of differential expression analysis is a large and complex one, with many techniques available to the potential user. These can be categorized into several methodological approaches, including:

- (1) Differential screening,
- (2) Subtractive hybridization (SH) (includes methods such as chemical cross-linking subtraction—CCLS, suppression-PCR subtractive hybridization—SSH, and representational difference analysis—RDA),
- (3) Differential display (DD),
- (4) Restriction endonuclease facilitated analysis (including serial analysis of gene expression—SAGE—and gene expression fingerprinting—GEF),
- (5) Gene expression arrays, and
- (6) Expressed sequence tag (EST) analysis.

The above approaches have been used successfully to isolate differentially expressed genes in different model systems. However, each method has its own subtle (and sometimes not so subtle) characteristics which incur various advantages and disadvantages. Accordingly, it is the purpose of this review to clarify the mechanistic principles underlying the main differential expression methods and to highlight some of the broader considerations and implications of this very powerful and increasingly popular technique. Specifically, we will concentrate on the so-called 'open' systems, namely those which do not require any knowledge of gene sequences and, therefore, are useful for isolating unknown genes. Two 'closed' systems (those utilising previously identified gene sequences), EST analysis and the use of DNA arrays, will also be considered briefly for completeness. Whilst emphasis will often be placed on suppression PCR subtractive hybridization (SSH, the approach employed in this laboratory), it is the aim of the authors to highlight, wherever possible, those areas of common interest to those who use, or intend to use, differential gene expression analysis.

### Differential cDNA library screening (DS)

Despite the development of multiple technological advances which have recently brought the field of gene expression profiling to the forefront of molecular analysis, recognition of the importance of differential gene expression and characterization of differentially expressed genes has existed for many years. One of the original approaches used to identify such genes was described 20 years ago by St John and Davis (1979). These authors developed a method, termed 'differential plaque filter

hybridization', which was used to isolate galactose-inducible DNA sequences from yeast. The theory is simple: a genomic DNA library is prepared from normal, unstimulated cells of the test organism/tissue and multiple filter replicas are prepared. These replica blots are probed with radioactively (or otherwise) labelled complex cDNA probes prepared from the control and test cell mRNA populations. Those mRNAs which are differentially expressed in the treated cell population will show a positive signal only on the filter probed with cDNA from the treated cells. Furthermore, labelled cDNA from different test conditions can be used to probe multiple blots, thereby enabling the identification of mRNAs which are only up-regulated under certain conditions. For example, St John and Davis (1979) screened replica filters with acetate-, glucose- and galactose-derived probes in order to obtain genes induced specifically by galactose metabolism. Although groundbreaking in its time this method is now considered insensitive and time-consuming, as up to 2 months are required to complete the identification of genes which are differentially expressed in the test population. In addition, there is no convenient way to check that the procedure has worked until the whole process has been completed.

#### Subtractive Hybridization (SH)

The developing concept of differential gene expression and the success of early approaches such as that described by St John and Davis (1979) soon gave rise to a search for more convenient methods of analysis. One of the first to be developed was SH, numerous variations of which have since been reported (see below). In general, this approach involves hybridization of mRNA/cDNA from one population (tester) to excess mRNA/cDNA from another (driver), followed by separation of the unhybridized tester fraction (differentially expressed) from the hybridized common sequences. This step has been achieved physically, chemically and through the use of selective polymerase chain reaction (PCR) techniques.

#### Physical separation

Original subtractive hybridization technology involved the physical separation of hybridized common species from unique single stranded species. Several methods of achieving this have been described, including hydroxyapatite chromatography (Sargent and Dawid 1983), avidin-biotin technology (Duguid and Dinauer 1990) and oligodT-latex separation (Hara *et al.* 1991). In the first approach, common mRNA species are removed by cDNA (from test cells)-mRNA (from control cells) subtractive hybridization followed by hydroxyapatite chromatography, as hydroxyapatite specifically adsorbs the cDNA-mRNA hybrids. The unabsorbed cDNA is then used either for the construction of a cDNA library of differentially expressed genes (Sargent and Dawid 1983, Schneider *et al.* 1988) or directly as a probe to screen a preselected library (Zimmerman *et al.* 1980, Davis *et al.* 1984, Hedrick *et al.* 1984). A schematic diagram of the procedure is shown in figure 1.

Less rigorous physical separation procedures coupled with sensitivity enhancing PCR steps were later developed as a means to overcome some of the problems encountered with the hydroxyapatite procedure. For example, Duguid and Dinauer (1990) described a method of subtraction utilizing biotin-affinity systems as a means to remove hybridized common sequences. In this process, both the control and tester mRNA populations are first converted to cDNA and an adaptor ('oligovector',

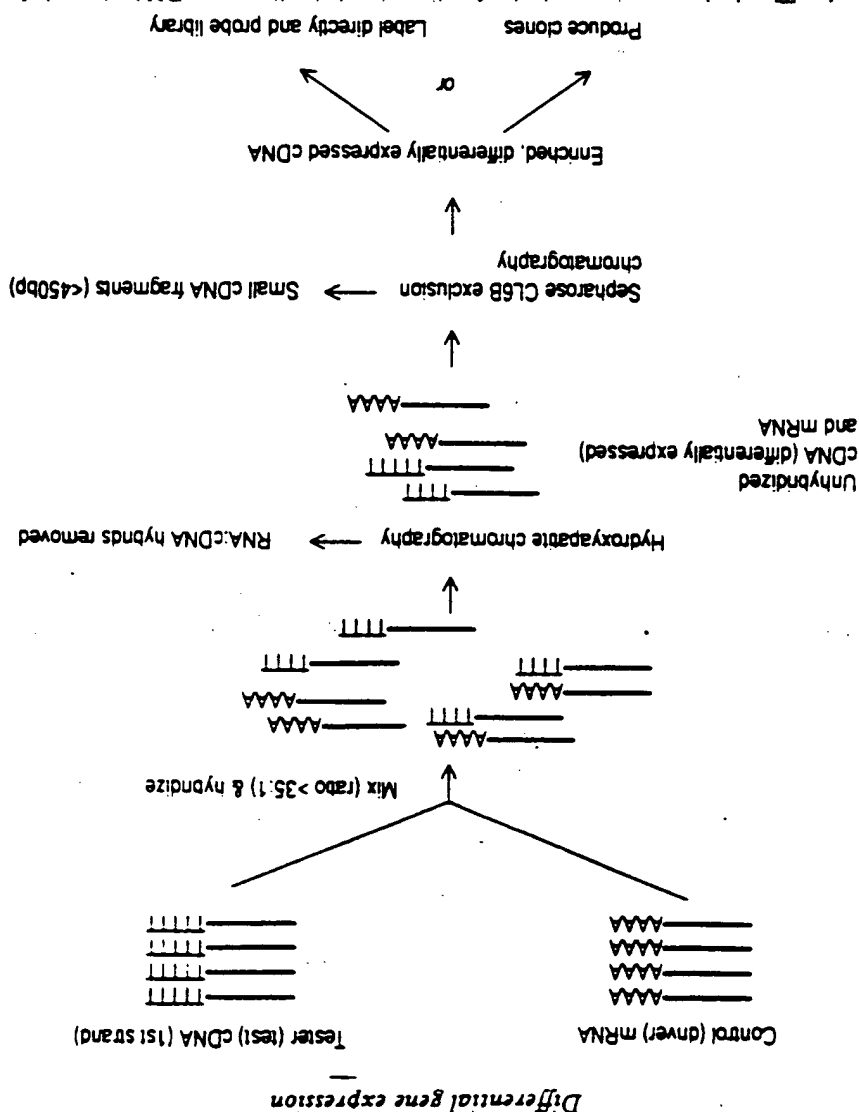
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containing a restriction site) ligated to both sides. Both populations are then amplified by PCR, but the driver cDNA population is subsequently digested with the adaptor-containing restriction endonuclease. This serves to cleave the oligo-control population and reduce the amplification potential of the control population. The digested control population is then biotinylated and an excess mixed with tester cDNA. Following denaturation and hybridization, the mix is applied to a biocytin column (streptavidin may also be used) to remove the control population, including heteroduplexes formed by annealing of common sequences from the tester population. The procedure is repeated several times following the addition of fresh

Figure 1. The hydroxyapatite method of subtractive hybridization. cDNA derived from the treated/ altered population is mixed with a large excess of mRNA from the control (driver) population. Following hybridization, mRNA-cDNA hybrids are removed by hydroxyapatite chromatography. The only cDNAs which remain are those which are differentially expressed in the treated/ altered population. In order to facilitate the recovery of full length clones, small cDNA fragments are removed by exclusion chromatography. The remaining cDNAs are then cloned into a vector for sequencing, or labelled and used directly to probe a library, as described by Sargent and Dawid (1983).



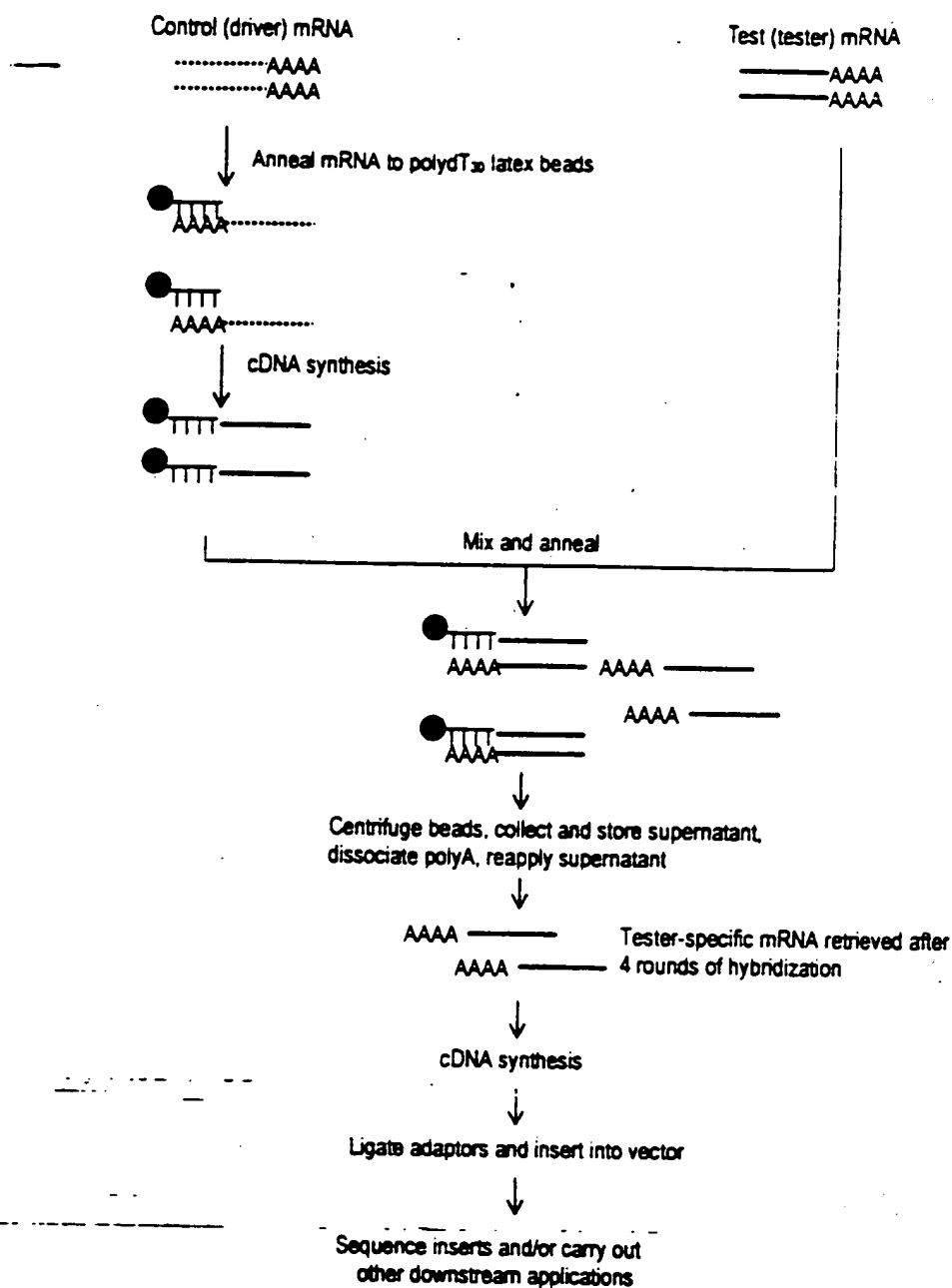


Figure 2. The use of oligodT<sub>30</sub> latex to perform subtractive hybridization. mRNA extracted from the control (driver) population is converted to anchored cDNA using polydT oligonucleotides attached to latex beads. mRNA from the treated/alterd (tester) population is repeatedly hybridized against an excess of the anchored driver cDNA. The final population of mRNA is tester specific and can be converted into cDNA for cloning and other downstream applications, as described by Hara et al. (1991).

control cDNA. In order to further enrich those species differentially expressed in the tester cDNA, the subtracted tester population is amplified by PCR following every second subtraction cycle. After six cycles of subtraction (three reamplification steps) the reaction mix is ligated into a vector for further analysis.

In a slightly different approach, Hara *et al.* (1991) utilized a method whereby oligo(dT<sub>30</sub>) primers attached to a latex substrate are used to first capture mRNA extracted from the control population. Following 1st strand cDNA synthesis, the RNA strand of the heteroduplexes is removed by heat denaturation and centrifugation (the cDNA-oligotex-dT<sub>30</sub> forms a pellet and the supernatant is removed). A quantity of tester mRNA is then repeatedly hybridized to the immobilized control (driver) cDNA (which is present in 20-fold excess). After several rounds of hybridization the only mRNA molecules left in the tester mRNA population are those which are not found in the driver cDNA-oligotex-dT<sub>30</sub> population. These tester-specific mRNA species are then converted to cDNA and, following the addition of adaptor sequences, amplified by PCR. The PCR products are then ligated into a vector for further analysis using restriction sites incorporated into the PCR primers. A schematic illustration of this subtraction process is shown in figure 2.

However, all these methods utilising physical separation have been described as inefficient due to the requirement for large starting amounts of mRNA, significant loss of material during the separation process and a need for several rounds of hybridization. Hence, new methods of differential expression analysis have recently been designed to eliminate these problems.

#### Chemical Cross-Linking Subtraction (CCLS)

In this technique, originally described by Hampson *et al.* (1992), driver mRNA is mixed with tester cDNA (1st strand only) in a ratio of > 20:1. The common sequences form cDNA:mRNA hybrids, leaving the tester specific species as single stranded cDNA. Instead of physically separating these hybrids, they are inactivated then synthesized from the remaining single stranded cDNA species (unreacted mRNA species remaining from the driver are not converted into probe material due to specificity of Sequenase T7 DNA polymerase used to make the probe) and used to screen a cDNA library made from the tester cell population. A schematic diagram of the system is shown in figure 3.

It has been shown that the differentially expressed sequences can be enriched at least 300-fold with one round of subtraction (Hampson *et al.* 1992), and that the technique should allow isolation of cDNAs derived from transcripts that are present at less than 50 copies per cell. This equates to genes at the low end of intermediate abundance (see table 1). The main advantages of the CCLS approach are that it is rapid, technically simple and also produces fewer false positives than other differential expression analysis methods. However, like the physical separation protocols, a major drawback with CCLS is the large amount of starting material required (at least 10 µg RNA). Consequently, the technique has recently been refined so that a renewable source of RNA can be generated. The degenerate random oligonucleotide primed (DROF) adaptation (Hampson *et al.* 1996, Hampson and Hampson 1997) uses random hexanucleotide sequences to prime solid phase-synthesized cDNA. Since each primer includes a T7 polymerase promoter sequence

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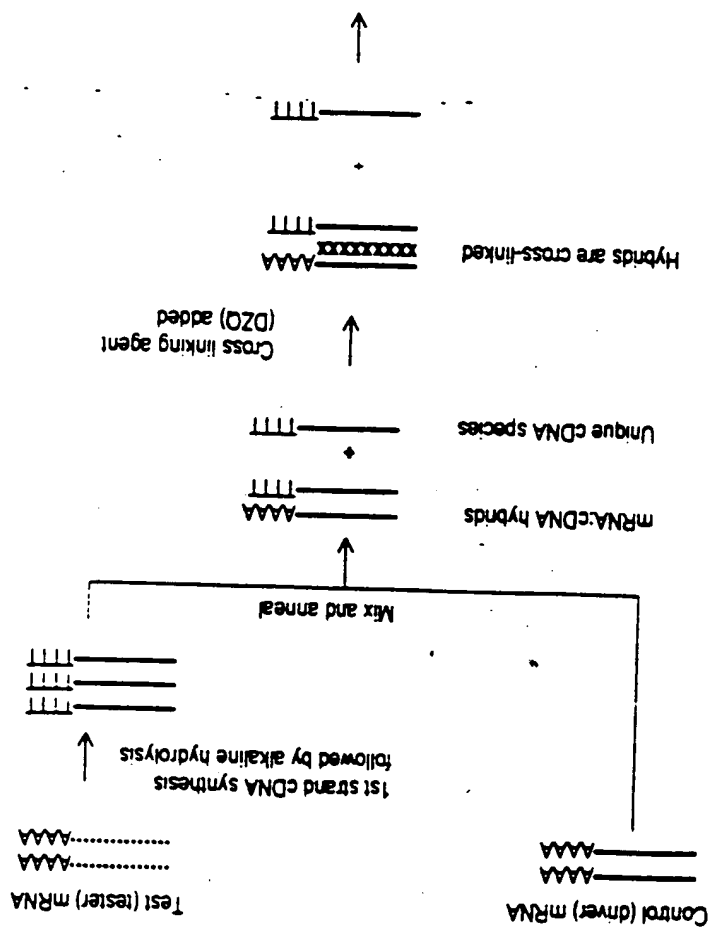


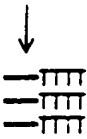
Figure 3. Chemical cross-linking subtraction. Excess driver mRNA is mixed with 1<sup>st</sup> strand tester cDNA. The common sequences form mRNA:cDNA hybrids which are cross-linked with 2,2'-dimethyl-1,4-benzodioxane (DZO) and the remaining cDNA sequences are differentially expressed in the tester population. Probes are made from these sequences using Sequenase 2.0 DNA polymerase, which lacks reverse transcriptase activity and, therefore, does not react with the remaining mRNA molecules from the driver. The labelled probes are then used to screen a cDNA library for clones of differentially expressed sequences. Adapted from Walter et al. (1996), with permission.

Table 1. The abundance of mRNA species and classes in a typical mammalian cell.

mRNA class	Copies of each species/cell	No. of mRNA species in class	Mean % of each species in class	Mean mass (ng) of each species/ $\mu$ g total RNA
Abundant	12 000	4	3.3	1.65
Intermediate	300	500	0.08	0.04
Rare	15	11 000	0.004	0.002

— Modified from Bertoli et al. (1995).

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at the 5' end, the final pool of random cDNA fragments is a PCR-renewable cDNA population which is representative of the expressed gene pool and can be used to synthesize sense RNA for use as driver material. Furthermore, if the final pool of random cDNA fragments is reamplified using biotinylated T7 primer and random hexamer, the product can be captured with streptavidin beads and the antisense strand eluted for use as tester. Since both target and driver can be generated from the same DROP product, subtraction can be performed in both directions (i.e. for up- and down-regulated species) between two different DROP products.

#### *Representational Difference Analysis (RDA)*

RDA of cDNA (Hubank and Schatz 1994) is an extension of the technique originally applied to genomic DNA as a means of identifying differences between two complex genomes (Lisitsyn *et al.* 1993). It is a process of subtraction and amplification involving subtractive hybridization of the tester in the presence of excess driver. Sequences in the tester that have homologues in the driver are rendered unamplifiable, whereas those genes expressed only in the tester retain the ability to be amplified by PCR. The procedure is shown schematically in figure 4.

In essence, the driver and tester mRNA populations are first converted to cDNA and amplified by PCR following the ligation of an adaptor. The adaptors are then removed from both populations and a new (different) adaptor ligated to the amplified tester population only. Driver and tester populations are next melted and hybridized together in a ratio of 100:1. Following hybridization, only tester:tester homohybrids have 5' adaptors at each end of the DNA duplex and can, thus, be filled in at both 3' ends. Hence, only these molecules are amplified exponentially during the subsequent PCR step. Although tester:driver heterohybrids are present, they only amplify in a linear fashion, since the strand derived from the driver has no adaptor to which the primer can bind. Driver:driver heterohybrids have no adaptors and, therefore, are not amplified. Single stranded molecules are digested with mung bean nuclease before a further PCR-enrichment of the tester:tester homohybrids. The adaptors on the amplified tester population are then replaced and the whole process repeated a further two or three times using an increasing excess of driver (Hubank and Shatz used a tester:driver ratio of 1:400, 1:80000 and 1:800000 for the second, third and fourth hybridizations, respectively). Different adaptors are ligated to the tester between successive rounds of hybridization and amplification to prevent the accumulation of PCR products that might interfere with subsequent amplifications. The final display is a series of differentially expressed gene products easily observable on an ethidium bromide gel.

The main advantages of RDA are that it offers a reproducible and sensitive approach to the analysis of differentially expressed genes. Hubank and Schatz (1994) reported that they were able to isolate genes that were differentially expressed in substantially less than 1% of the cells from which the tester is derived. Perhaps the main drawback is that multiple rounds of ligation, hybridization, amplification and digestion are required. The procedure is, therefore, lengthier than many other differential display approaches and provides more opportunity for operator-induced error to occur. Although the generation of false positives has been noted, this has been solved to some degree by O'Neill and Sinclair (1997) through the use of HPLC-purified adaptors. These are free of the truncated adaptors which appear to be a major source of the false positive bands. A very similar technique to RDA, termed linker capture subtraction (LCS) was described by Yang and Sytowski (1996).

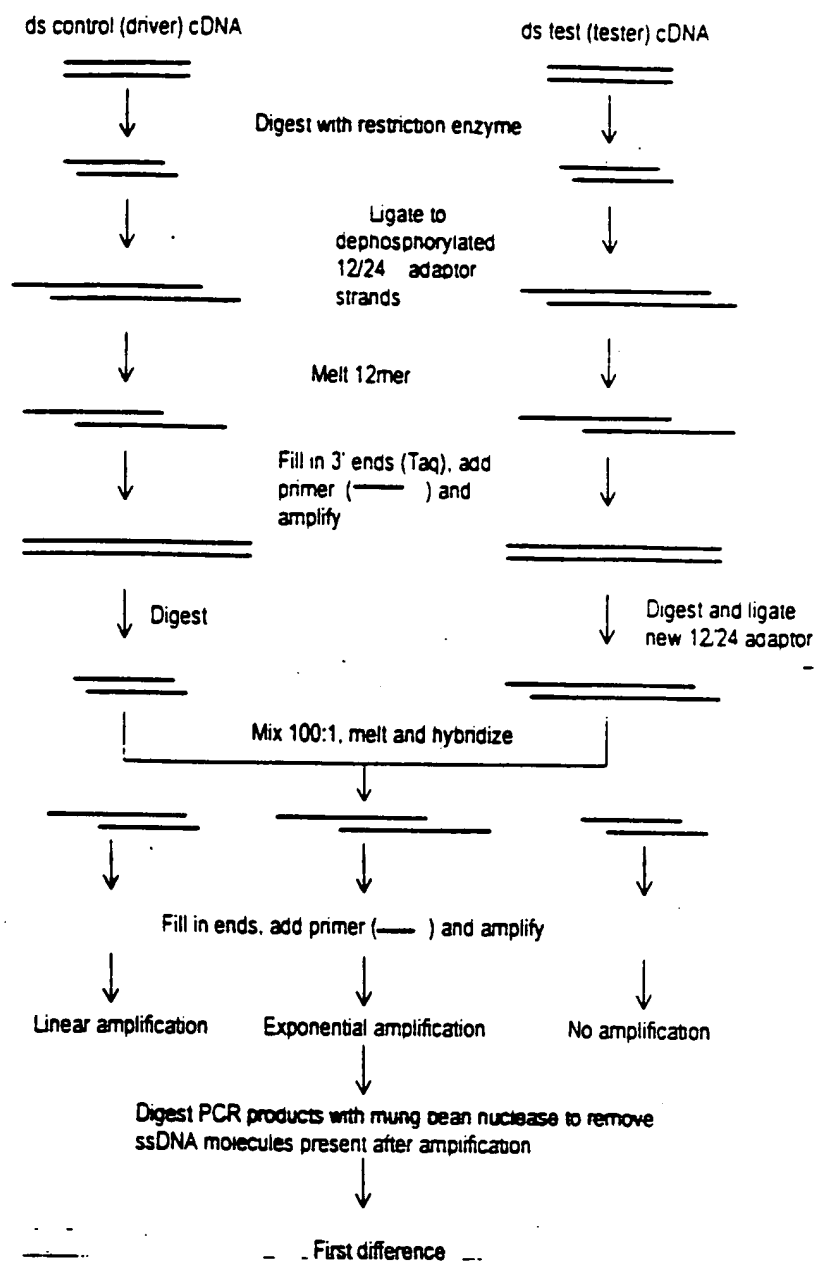


Figure 4. The representational difference analysis (RDA) technique. Driver and tester cDNA are digested with a 4-cutter restriction enzyme such as *DpnII*. The 1<sup>st</sup> set of 12/24 adaptor strands (oligonucleotides) are ligated to each other and the digested cDNA products. The 12mer is subsequently melted away and the 3' ends filled in using Taq DNA polymerase. Each cDNA population is then amplified using PCR, following which the 1<sup>st</sup> set of adaptors is removed with *DpnII*. A second set of 12/24 adaptor strands is then added to the amplified tester cDNA population, after which the tester is hybridized against a large excess of driver. The 12mer adaptors are melted and the 3' ends filled in as before. PCR is carried out with primers identical to the new 24mer adaptor. Thus, the only hybridization products which are exponentially amplified are those which are tester:tester combinations. Following PCR, ssDNA products are removed with mung bean nuclease, leaving the 'first difference product'. This is digested and a third set of 12/24 adaptors added before repeating the subtraction process from the hybridization stage. The process is repeated to the 3<sup>rd</sup> or 4<sup>th</sup> difference product, as described by Lisitsyn *et al.* (1993) and Hubank and Schatz (1994).

*Suppression PCR Subtractive Hybridization (SSH)*

The most recent adaptation of the SH approach to differential expression analysis was first described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996). They reported that a 1000–5000 fold enrichment of rare cDNAs (equivalent to isolating mRNAs present at only a few copies per cell) can be obtained without the need for multiple hybridizations/subtractions. Instead of physical or chemical removal of the common sequences, a PCR-based suppression system is used (see figure 5).

In SSH, excess driver cDNA is added to two portions of the tester cDNA which have been ligated with different adaptors. A first round of hybridization serves to enrich differentially expressed genes and equalize rare and abundant messages. Equalization occurs since reannealing is more rapid for abundant molecules than for rarer molecules due to the second order kinetics of hybridization (James and Higgins 1985). The two primary hybridization mixes are then mixed together in the presence of excess driver and allowed to hybridize further. This step permits the annealing of single stranded complementary sequences which did not hybridize in the primary hybridization, and in doing so generates templates for PCR amplification. Although there are several possible combinations of the single stranded molecules present in the secondary hybridization mix, only one particular combination (differentially expressed in the tester cDNA composed of complementary strands having different adaptors) can amplify exponentially.

Having obtained the final differential display, two options are available if cloning of cDNAs is desired. One is to transform the whole of the final PCR reaction into competent cells. Transformed colonies can then be isolated and their inserts characterized by sequencing, restriction analysis or PCR. Alternatively, the final PCR products can be resolved on a gel and the individual bands excised, reamplified and cloned. The first approach is technically simpler and less time consuming. However, ligation/transformation reactions are known to be biased towards the cloning of smaller molecules, and so the final population of clones will probably not contain a representative selection of the larger products. In addition, although equalization theoretically occurs, observations in this laboratory suggest that this is by no means perfectly accomplished. Consequently, some gene species are present in a higher number than others and this will be represented in the final population of clones. Thus, in order to obtain a substantial proportion of those gene species that actually demonstrate differential expression in the tester population, the number of clones that will have to be screened after this step may be substantial. The second approach is initially more time consuming and technically demanding. However, it would appear to offer better prospects for cloning larger and low abundance gel products. In addition, one can incorporate a screening step that differentiates different products of different sequences but of the same size (HA-staining, see later). In this way, a good idea of the final number of clones to be isolated and identified can be achieved.

An alternative (or even complementary) approach is to use the final differential display reaction to screen a cDNA library to isolate full length clones for further characterization, or a DNA array (see later) to quickly identify known genes. SSH has been used in this laboratory to begin characterization of the short-term gene expression profiles of enzyme-inducers such as phenobarbital (Rockett *et al.* 1997) and Wy-14,643 (Rockett *et al.* unpublished observations). The isolation of differentially expressed genes in this manner enables the construction of a fingerprint

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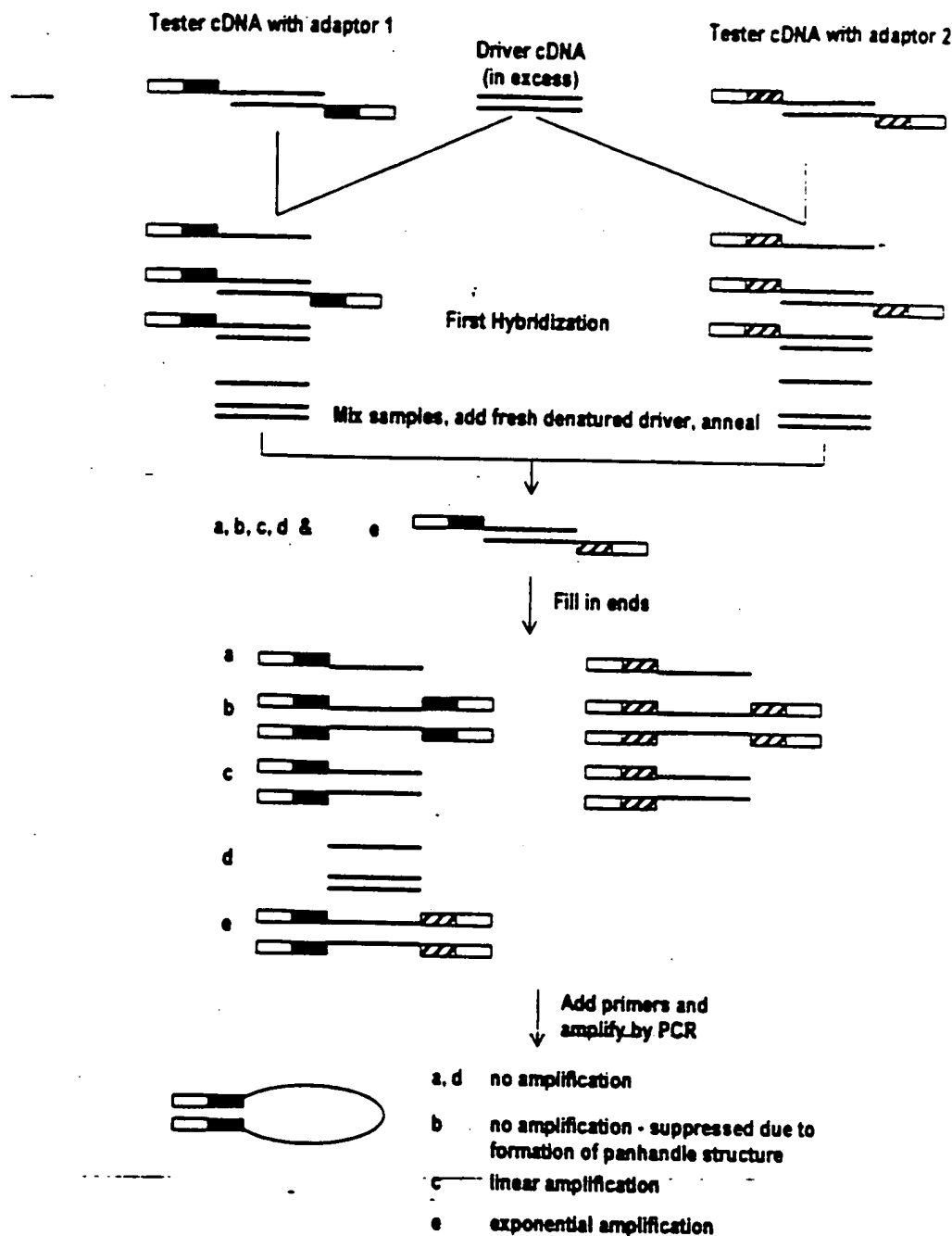


Figure 5. PCR-select cDNA subtraction. In the primary hybridization, an excess of driver cDNA is added to each tester cDNA population. The samples are heat denatured and allowed to hybridize for between 3 and 8 h. This serves two purposes: (1) to equalize rare and abundant molecules; and (2) to enrich for differentially expressed sequences—cDNAs that are not differentially expressed form type c molecules with the driver. In the secondary hybridization, the two primary hybridizations are mixed together without denaturing. Fresh denatured driver can also be added at this point to allow further enrichment of differentially expressed sequences. Type e molecules are formed in this secondary hybridization which are subsequently amplified using two rounds of PCR. The final products can be visualized on an agarose gel, labelled directly or cloned into a vector for downstream manipulation. As described by Diatchenko *et al.* (1996) and Gurakaya *et al.* (1996), with permission.

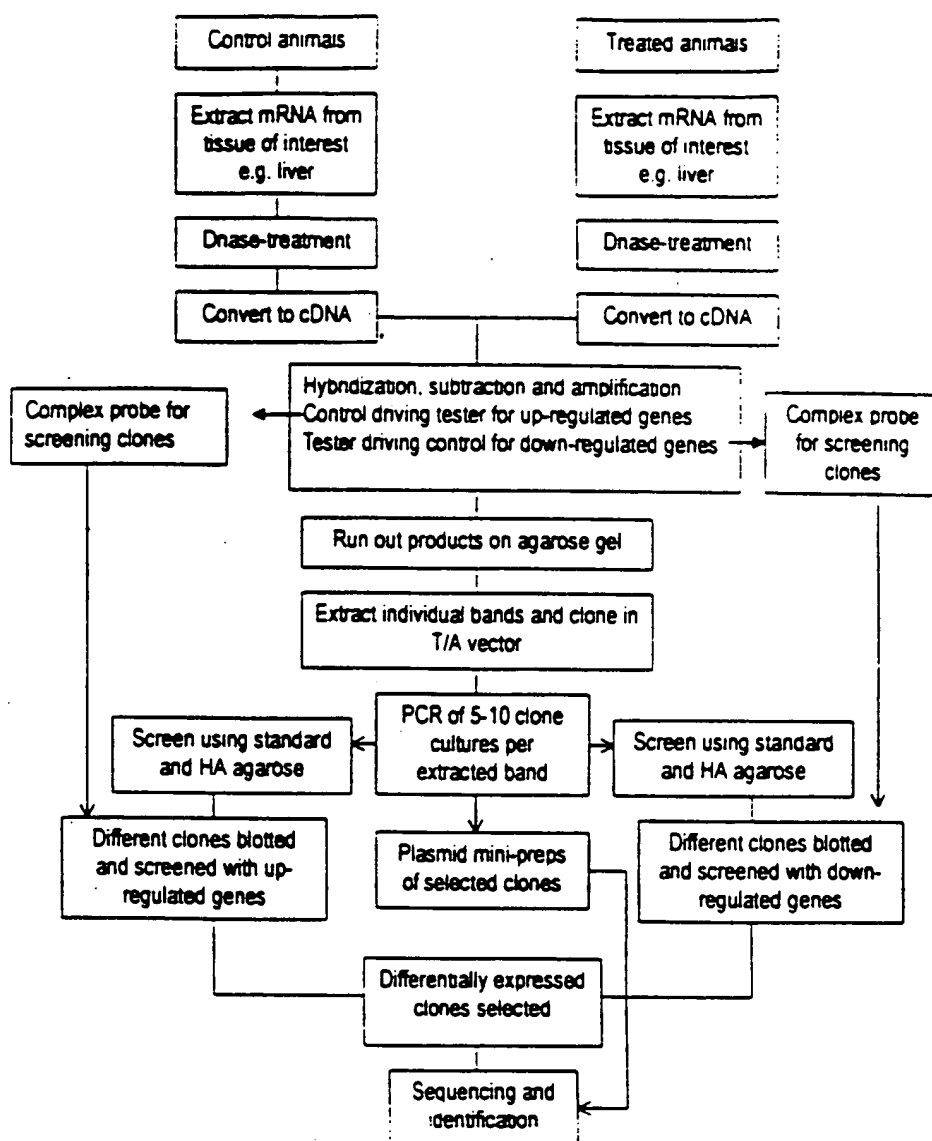
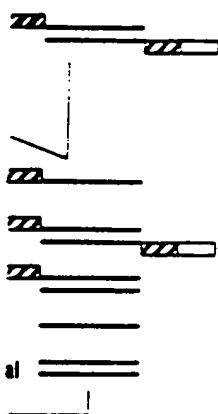


Figure 6. Flow diagram showing method used in this laboratory to isolate and identify clones of genes which are differentially expressed in rat liver following short term exposure to the enzyme inducers, phenobarbital and Wy-14,643.

of expressed genes which are unique to each compound and time/dose point. Such information could be useful in short-term characterization of the toxic potential of new compounds by comparing the gene-expression profiles they elicit with those produced by known inducers. Figure 6 shows a flow diagram of the method used to isolate, verify and clone differentially expressed genes, and figure 7 shows expression profiles obtained from a typical SSH experiment. Subsequent sub-cloning of the individual bands, sequencing and gene data base interrogation reveals many genes which are either up- or down-regulated by phenobarbital in the rat (tables 2 and 3).

One of the advantages in using the SSH approach is that no prior knowledge is required of which specific genes are up/down-regulated subsequent to xenobiotic

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n excess of driver cDNA is red and allowed to hybridize and abundant molecules; and not differentially expressed. In hybridization, the two primary red driver can also be added. Sequences. Type e molecules amplified using two rounds of hybridization directly or cloned into a vector. (1996) and Gurakaya

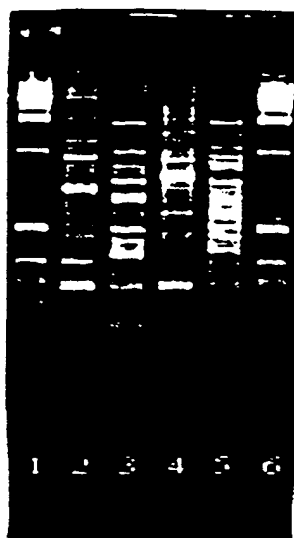


Figure 7. SSH display patterns obtained from rat liver following 3-day treatment with Wy,14,643 or phenobarbital. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane: 1—1kb ladder; 2—genes upregulated following Wy,14,643 treatment; 3—genes downregulated following Wy,14,643 treatment; 4—genes upregulated following phenobarbital treatment; 5—genes downregulated following phenobarbital treatment; 6—1kb ladder. Reproduced from Rockett *et al.* (1997), with permission.

exposure, and an almost complete complement of genes are obtained. For example, the peroxisome proliferator and non-genotoxic hepatocarcinogen Wy,14,643, up-regulates at least 28 genes and down-regulates at least 15 in the rat (a sensitive species) and produces 48 up- and 37 down-regulated genes in the guinea pig, a resistant species (Rockett, Swales, Esda and Gibson, unpublished observations). One of these genes, CD81, was up-regulated in the rat and down-regulated in the guinea pig following Wy-14,643 treatment. CD81 (alternatively named TAPA-1) is a widely expressed cell surface protein which is involved in a large number of cellular processes including adhesion, activation, proliferation and differentiation (Levy *et al.* 1998). Since all of these functions are altered to some extent in the phenomena of hepatomegaly and non-genotoxic hepatocarcinogenesis, it is intriguing, and probably mechanistically-relevant, that CD81 expression is differentially regulated in a resistant and susceptible species. However, the down-side of this approach is that the majority of genes can be sequenced and matched to database sequences, but the latter are predominantly expressed sequence tags or genes of completely unknown function, thus partially obscuring a realistic overall assessment of the critical genes of genuine biological interest. Notwithstanding the lack of complete functional identification of altered gene expression, such gene profiling studies essentially provides a 'molecular fingerprint' in response to xenobiotic challenge, thereby serving as a mechanistically-relevant platform for further detailed investigations.

#### Differential Display (DD)

Originally described as 'RNA fingerprinting by arbitrarily primed PCR' (Liang and Pardee 1992) this method is now more commonly referred to as 'differential

Table 2. Genes up-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
5 (1300)	93.5%	CYP2B1
7 (1000)	95.1%	Preproalbumin Serum albumin mRNA
8 (950)	98.3%	NCI-CGAP-Pr1 <i>H. sapiens</i> (EST)
10 (850)	95.7%	CYP2B1
11 (800)	Clone 1 94.9%	CYP2B1
	Clone 2 75.3%	CYP2B2
12 (750)	93.8%	TRPM-2 mRNA Sulfated glycoprotein
15 (600)	92.9%	Preproalbumin Serum albumin mRNA
16 (55)	Clone 1 95.2%	CYP2B1
	Clone 2 93.6%	Haptoglobin mRNA partial alpha
21 (350)	99.3%	18S, 5.8S & 28S rRNA

Bands 1-4, 6, 9, 13, 14, and 17-20 are shown to be false positives by dot blot analysis and, therefore, are not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are up-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

Table 3. Genes down-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
1 (1500)	95.3%	3-oxoacyl-CoA thiolase
2 (1200)	92.3%	Hemopoxin mRNA
3 (1000)	91.7%	Alpha-2u-globulin mRNA
7 (700)	Clone 1 77.2%	<i>M. musculus</i> C1 inhibitor
	Clone 2 94.5%	Electron transfer flavoprotein
	Clone 3 91.0%	<i>M. musculus</i> Topoisomerase 1 (Topo 1).
8 (650)	Clone 1 86.9%	Soares 2NbMT <i>M. musculus</i> (EST)
	Clone 2 96.2%	Alpha-2u-globulin (s-type) mRNA
9 (600)	Clone 1 86.9%	Soares mouse NML <i>M. musculus</i> (EST)
	Clone 2 82.0%	Soares p3NMF 19.5 <i>M. musculus</i> (EST)
10 (550)	73.8%	Soares mouse NML <i>M. musculus</i> (EST)
11 (525)	95.7%	NCI-CGAP-Pr1 <i>H. sapiens</i> (EST)
12 (375)	100.0%	Ribosomal protein
13 (23)	Clone 1 97.2%	Soares mouse embryo NbME135 (EST)
	Clone 2 100.0%	Fibrinogen B-beta-chain
	Clone 3 100.0%	Apolipoprotein E gene
14 (170)	96.0%	Soares p3NMF19.5 <i>M. musculus</i> (EST)
15 (140)	97.3%	Stratagene mouse testis (EST)
Others: (300)	96.7%	<i>R. norvegicus</i> RASP 1 mRNA
(275)	93.1%	Soares mouse mammary gland (EST)

EST = Expressed sequence tag. Bands 4-6 were shown to be false positives by dot blot analysis and, therefore, were not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are down-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

display' (DD). In this method, all the mRNA species in the control and treated cell populations are amplified in separate reactions using reverse transcriptase-PCR (RT-PCR). The products are then run side-by-side on sequencing gels. Those bands which are present in one display only, or which are much more intense in one

ment with WY-14,643 or was used to generate the (Clontech). Lane: 1—1kb es downregulated following bital treatment; 5—genes eproduced from Rockett *et*

btained. For example, ogen Wy,14,643, up- in the rat (a sensitive s in the guinea pig, a plished observations). down-regulated in the sly named TAPA-1) is rge number of cellular ifferentiation (Levy *et* ent in the phenomena it is intriguing, and ifferentially regulated de of this approach is atabase sequences, but genes of completely all assessment of the g the lack of complete gene profiling studies xenobiotic challenge, for further detailed

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display compared to the other, are differentially expressed and may be recovered for further characterization. One advantage of this system is the speed with which it can be carried out—2 days to obtain a display and as little as a week to make and identify clones.

Two commonly used variations are based on different methods of priming the reverse transcription step (figure 8). One is to use an oligo dT with a 2-base 'anchor' at the 3'-end, e.g. 5' (dT<sub>11</sub>)CA 3' (Liang and Pardee 1992). Alternatively, an arbitrary primer may be used for 1st strand cDNA synthesis (Welsh *et al.* 1992). This variant of RNA fingerprinting has also been called 'RAP' (RNA Arbitrarily Primed)-PCR. One advantage of this second approach is that PCR products may be derived from anywhere in the RNA, including open reading frames. In addition, it can be used for mRNAs that are not polyadenylated, such as many bacterial mRNAs (Wong and McClelland 1994). In both cases, following reverse transcription and denaturation, second strand cDNA synthesis is carried out with an arbitrary primer (*arbitrary* primers have a single base at each position, as compared to *random* primers, which contain a mixture of all four bases at each position). The resulting PCR, thus, produces a series of products which, depending on the system (primer length and composition, polymerase and gel system), usually includes 50–100 products per primer set (Band and Sager 1989). When a combination of different dT-anchors and arbitrary primers are used, almost all mRNA species from a cell can be amplified. When the cDNA products from two different populations are analysed side by side on a polyacrylamide gel, differences in expression can be identified and the appropriate bands recovered for cloning and further analysis.

Although DD is perhaps the most popular approach used today for identifying differentially expressed genes, it does suffer from several perceived disadvantages:

- (1) It may have a strong bias towards high copy number mRNAs (Bertioli *et al.* 1995), although this has been disputed (Wan *et al.* 1996) and the isolation of very low abundance genes may be achieved in certain circumstances (Guimeraes *et al.* 1995a).
- (2) The cDNAs obtained often only represent the extreme 3' end of the mRNA (often the 3'-untranslated region), although this may not always be the case (Guimeraes *et al.* 1995a). Since the 3' end is often not included in Genbank and shows variation between organisms, cDNAs identified by DD cannot always be matched with their genes, even if they have been identified.
- (3) The pattern of differential expression seen on the display often cannot be reproduced on Northern blots, with false positives arising in up to 70% of cases (Sun *et al.* 1994). Some adaptations have been shown to reduce false positives, including the use of two reverse transcriptases (Sung and Denman 1997), comparison of uninduced and induced cells over a time course (Burn *et al.* 1994) and comparison of DDPCR-products from two uninduced and two induced lines (Sompayrac *et al.* 1995). The latter authors also reported that the use of cytoplasmic RNA rather than total RNA reduces false positives arising from nuclear RNA that is not transported to the cytoplasm.

Further details of the background, strengths and weaknesses of the DD technique can be obtained from a review by McClelland *et al.* (1996) and from articles by Liang *et al.* (1995) and Wan *et al.* (1996).

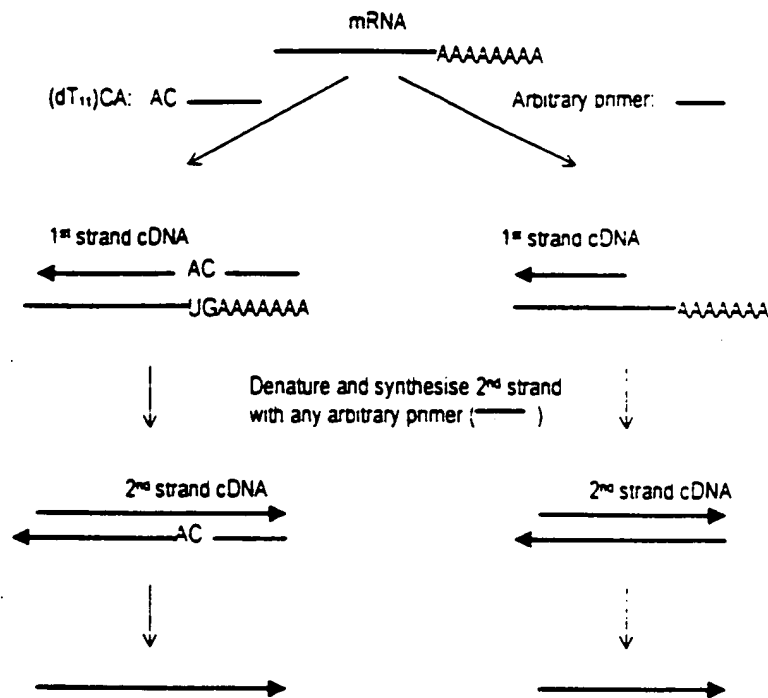


Figure 8. Two approaches to differential display (DD) analysis. 1<sup>st</sup> strand synthesis can be carried out either with a polydT<sub>11</sub>NN primer (where N = G, C or A) or with an arbitrary primer. The use of different combinations of G, C and A to anchor the first strand polydT primer enables the priming of the majority of polyadenylated mRNAs. Arbitrary primers may hybridize at none, one or more places along the length of the mRNA, allowing 1<sup>st</sup> strand cDNA synthesis to occur at none, one or more points in the same gene. In both cases, 2<sup>nd</sup> strand synthesis is carried out with an arbitrary primer. Since these arbitrary primers for the 2<sup>nd</sup> strand may also hybridize to the 1<sup>st</sup> strand cDNA in a number of different places, several different 2<sup>nd</sup> strand products may be obtained from one binding point of the 1<sup>st</sup> strand primer. Following 2<sup>nd</sup> strand synthesis, the original set of primers is used to amplify the second strand products, with the result that numerous gene sequences are amplified.

### Restriction endonuclease-facilitated analysis of gene expression

#### Serial Analysis of Gene Expression (SAGE)

A more recent development in the field of differential display is SAGE analysis (Velculescu *et al.* 1995). This method uses a different approach to those discussed so far and is based on two principles. Firstly, in more than 95% of cases, short nucleotide sequences ('tags') of only nine or 10 base pairs provide sufficient information to identify their gene of origin. Secondly, concatenation (linking together in a series) of these tags allows sequencing of multiple cDNAs within a single clone. Figure 9 shows a schematic representation of the SAGE process. In this procedure, double stranded cDNA from the test cells is synthesized with a biotinylated polydT primer. Following digestion with a commonly cutting (4bp recognition sequence) restriction enzyme ('anchoring enzyme'), the 3' ends of the cDNA population are captured with streptavidin beads. The captured population is

split into two and different adaptors ligated to the 5' ends of each group. Incorporated into the adaptors is a recognition sequence for a type IIS restriction enzyme—one which cuts DNA at a defined distance (< 20 bp) from its recognition sequence. Hence, following digestion of each captured cDNA population with the IIS enzyme, the adaptors plus a short piece of the captured cDNA are released. The two populations are then ligated and the products amplified. The amplified products are cleaved with the original anchoring enzyme, religated (concatomers are formed in the process) and cloned. The advantage of this system is that hundreds of gene tags can be identified by sequencing only a few clones. Furthermore, the number of times a given transcript is identified is a quantitative measurement of that gene's abundance in the original population, a feature which facilitates identification of differentially expressed genes in different cell populations.

Some disadvantages of SAGE analysis include the technical difficulty of the method, a large amount of accurate sequencing is required, biased towards abundant mRNAs, has not been validated in the pharmaco/toxicogenomic setting and has only been used to examine well known tissue differences to date.

#### *Gene Expression Fingerprinting (GEF)*

A different capture/restriction digest approach for isolating differentially expressed genes has been described by Ivanova and Belyavsky (1995). In this method, RNA is converted to cDNA using biotinylated oligo(dT) primers. The cDNA population is then digested with a specific endonuclease and captured with magnetic streptavidin microbeads to facilitate removal of the unwanted 5' digestion products. The use of restricted 3'-ends alone serves to reduce the complexity of the cDNA fragment pool and helps to ensure that each RNA species is represented by not more than one restriction product. An adaptor is ligated to facilitate subsequent amplification of the captured population. PCR is carried out with one adaptor-specific and one biotinylated polydT primer. The reamplified population is recaptured and the non-biotinylated strands removed by alkaline dissociation. The non-biotinylated strand is then resynthesized using a different adaptor-specific primer in the presence of a radiolabelled dNTP. The labelled immobilized 3' cDNA ends are next sequentially treated with a series of different restriction endonucleases and the products from each digestion analysed by PAGE. The result is a fingerprint composed of a number of ladders (equal to the number of sequential digests used). By comparing test versus control fingerprints, it is possible to identify differentially expressed products which can then be isolated from the gel and cloned. The advantages of this procedure are that it is very robust and reproducible, and the authors estimate that 80–93% of cDNA molecules are involved in the final fingerprint. The disadvantage is that polyacrylamide gels can rarely resolve more than 300–400 bands, which compares poorly to the 1000 or more which are estimated to be produced in an average experiment. The use of 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991) may help to overcome this problem.

A similar method for displaying restriction endonuclease fragments was later described by Prashar and Weissman (1996). However, instead of sequential digestion of the immobilized 3'-terminal cDNA fragments, these authors simply compared the profiles of the control and treated populations without further manipulation.

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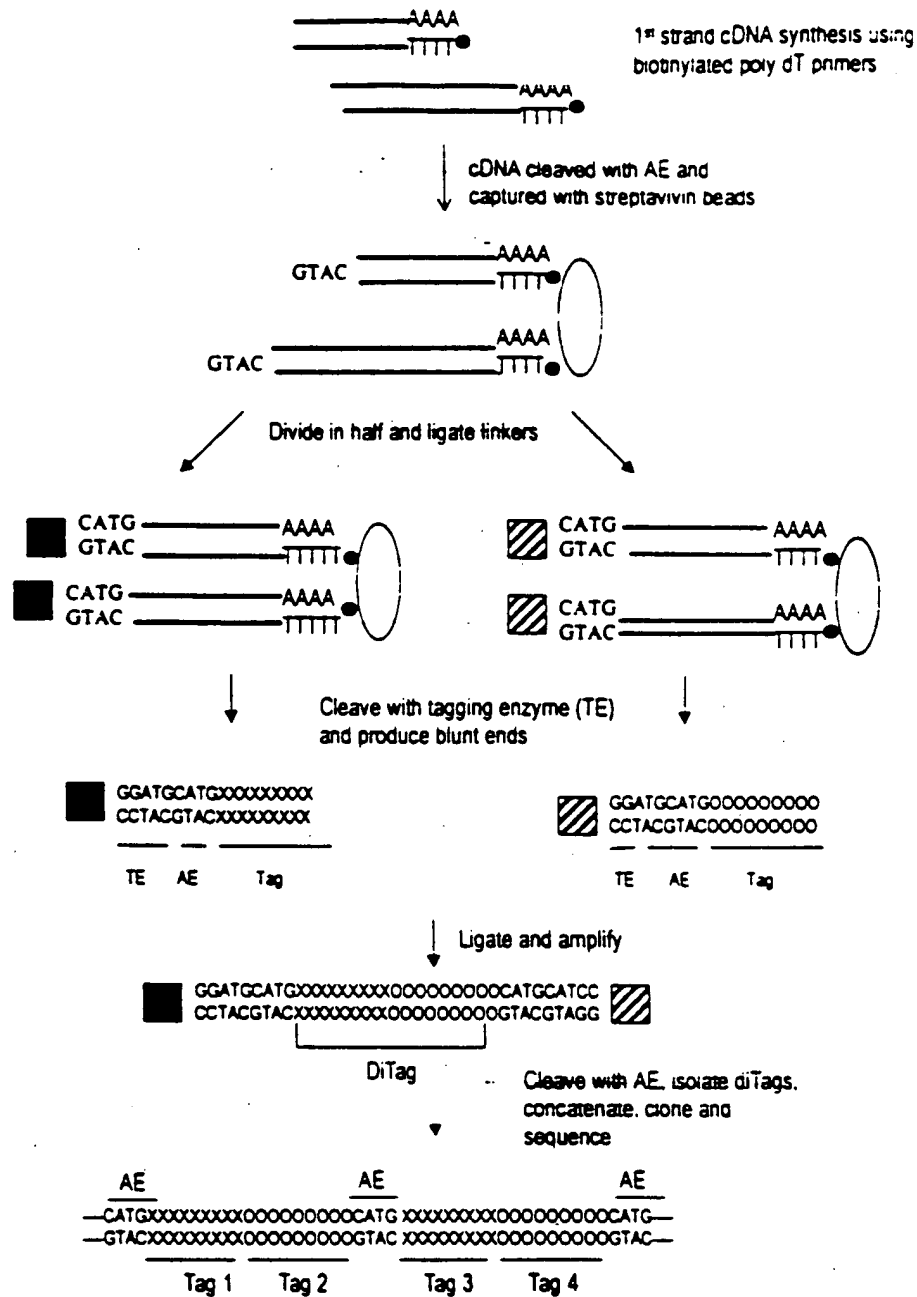


Figure 9. Serial analysis of gene expression (SAGE) analysis. cDNA is cleaved with an anchoring enzyme (AE) and the 3' ends captured using streptavidin beads. The cDNA pool is divided in half and each portion ligated to a different linker, each containing a type IIS restriction site (tagging enzyme, TE). Restriction with the type IIS enzyme releases the linker plus a short length of cDNA (XXXXXX and OOOOO indicate nucleotides of different tags). The two pools of tags are then ligated and amplified using linker-specific primers. Following PCR, the products are cleaved with the AE and the di-tags isolated from the linkers using PAGE. The di-tags are then ligated (during which process, concatenation occurs) and cloned into a vector of choice for sequencing. After Velculescu *et al.* (1995), with permission.

### DNA arrays

'Open' differential display systems are cumbersome in that it takes a great deal of time to extract and identify candidate genes and then confirm that they are indeed up- or down-regulated in the treated compared to the control tissue. Normally, the latter process is carried out using Northern blotting or RT-PCR. Even so, each of the aforementioned steps produce a bottleneck to the ultimate goal of rapid analysis of gene expression. These problems will likely be addressed by the development of so-called DNA arrays (e.g. Gress *et al.* 1992, Zhao *et al.* 1995, Schena *et al.* 1996), the introduction of which has signalled the next era in differential gene expression analysis. DNA arrays consist of a gridded membrane or glass 'chips' containing hundreds or thousands of DNA spots, each consisting of multiple copies of part of a known gene. The genes are often selected based on previously proven involvement in oncogenesis, cell cycling, DNA repair, development and other cellular processes. They are usually chosen to be as specific as possible for each gene and animal species. Human and mouse arrays are already commercially available and a few companies will construct a personalized array to order, for example Clontech Laboratories and Research Genetics Inc. The technique is rapid in that hundreds or even thousands of genes can be spotted on a single array, and that mRNA/cDNA from the test populations can be labelled and used directly as probe. When analysed with appropriate hardware and software, arrays offer a rapid and quantitative means to assess differences in gene expression between two cell populations. Of course, there can only be identification and quantitation of those genes which are in the array (hence the term 'closed' system). Therefore, one approach to elucidating the molecular mechanisms involved in a particular disease/development system may be to combine an open and closed system—a DNA array to directly identify and quantitate the expression of known genes in mRNA populations, and an open system such as SSH to isolate unknown genes which are differentially expressed.

One of the main advantages of DNA arrays is the huge number of gene fragments which can be put on a membrane—some companies have reported gridding up to 60000 spots on a single glass 'chip' (microscope slide). These high density chip-based micro-arrays will probably become available as mass-produced off-the-shelf items in the near future. This should facilitate the more rapid determination of differential expression in time and dose-response experiments. Aside from their high cost and the technical complexities involved in producing and probing DNA arrays, the main problem which remains, especially with the newer micro-array (gene-chip) technologies, is that results are often not wholly reproducible between arrays. However, this problem is being addressed and should be resolved within the next few years.

### EST databases as a means to identify differentially expressed genes

Expressed sequence tags (ESTs) are partial sequences of clones obtained from cDNA libraries. Even though most ESTs have no formal identity (putative identification is the best to be hoped for), they have proven to be a rapid and efficient means of discovering new genes and can be used to generate profiles of gene-expression in specific cells. Since they were first described by Adams *et al.* (1991), there has been a huge explosion in EST production and it is estimated that there are now well over a million such sequences in the public domain, representing over half

of all human genes (Hillier *et al.* 1996). This large number of freely available sequences (both sequence information and clones are normally available royalty-free from the originators) has enabled the development of a new approach towards differential gene expression analysis as described by Vasmataz *et al.* (1998). The approach is simple in theory: EST databases are first searched for genes that have a number of related EST sequences from the target tissue of choice, but none or few from non-target tissue libraries. Programmes to assist in the assembly of such sets of overlapping data may be developed in-house or obtained privately or from the internet. For example, the Institute for Genomic Research (TIGR, found at <http://www.tigr.org>) provides many software tools free of charge to the scientific community. Included amongst these is the TIGR assembler (Sutton *et al.* 1995), a tool for the assembly of large sets of overlapping data such as ESTs, bacterial artificial chromosomes (BAC)s, or small genomes. Candidate EST clones representing different genes are then analysed using RNA blot methods for size and tissue specificity and, if required, used as probes to isolate and identify the full length cDNA clone for further characterization. In practice however, the method is rather more involved, requiring bioinformatic and computer analysis coupled with confirmatory molecular studies. Vasmataz *et al.* (1998) have described several problems in this fledgling approach, such as separating highly homologous sequences derived from different genes and an overemphasis of specificity for some EST sequences. However, since these problems will largely be addressed by the development of more suitable computer algorithms and an increased completeness of the EST database, it is likely that this approach to identifying differentially expressed genes may enjoy more patronage in the future.

### Problems and potential of differential expression techniques

#### *The holistic or single cell approach?*

When working with *in vivo* models of differential expression, one of the first issues to consider must be the presence of multiple cell types in any given specimen. For example, a liver sample is likely to contain not only hepatocytes, but also (potentially) Ito cells, bile ductule cells, endothelial cells, various immune cells (e.g. lymphocytes, macrophages and Kupffer cells) and fibroblasts. Other tissues will each have their own distinctive cell populations. Also, in the case of neoplastic tissue, there are almost always normal, hyperplastic and/or dysplastic cells present in a sample. One must, therefore, be aware that genes obtained from a differential display experiment performed on an animal tissue model may not necessarily arise exclusively from the intended 'target' cells, e.g. hepatocytes/neoplastic cells. If appropriate, further analyses using immunohistochemistry, *in situ* hybridization or *in situ* RT-PCR should be used to confirm which cell types are expressing the gene(s) of interest. This problem is probably most acute for those studying the differential expression of genes in the development of different cell types, where there is a need to examine homologous cell populations. The problem is now being addressed at the National Cancer Institute (Bethesda, MD, USA) where new microdissection techniques have been employed to assist in their gene analysis programme, the Cancer Genome Anatomy Project (CGAP) (For more information see web site: <http://www.ncbi.nlm.nih.gov/ncicgap/intro.html>). There are also separation techniques available that utilise cell-specific antigens as a means to isolate target cells,

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e.g. fluorescence activated cell sorting (FACS) (Dunbar *et al.* 1998, Kas-Deelen *et al.* 1998) and magnetic bead technology (Richard *et al.* 1998, Rogler *et al.* 1998).

However, those taking a holistic approach may consider this issue unimportant. There is an equally appropriate view that all those genes showing altered expression within a compromised tissue should be taken into consideration. After all, since all tissues are complex mixes of different, interacting cell types which intimately regulate each other's growth and development, it is clear that each cell type could in some way contribute (positively or negatively) towards the molecular mechanisms which lie behind responses to external stimuli or neoplastic growth. It is perhaps then more informative to carry out differential display experiments using *in vivo* as opposed to *in vitro* models, where uniform populations of identical cells probably represent a partial, skewed or even inaccurate picture of the molecular changes that occur.

The incidence and possible implications of inter-individual biological variation should be considered in any approach where whole animal models are being used. It is clear that individuals (humans and animals) respond in different ways to identical stimuli. One of the best characterized examples is the debrisoquine oxidation polymorphism, which is mediated by cytochrome CYP2D6 and determines the pharmacokinetics of many commonly prescribed drugs (Lennard 1993, Meyer and Zanger 1997). The reasons for such differences are varied and complex, but allelic variations, regulatory region polymorphisms and even physical and mental health can all contribute to observed differences in individual responses. Careful thought should, therefore, be given to the specific objectives of the study and to the possible value of pooling starting material (tissue/mRNA). The effect of this can be beneficial through the ironing out of exaggerated responses and unimportant minor fluctuations of (mechanistically) irrelevant genes in individual animals, thus providing a clearer overall picture of the general molecular mechanisms of the response. However, at the same time such minor variations may be of utmost importance in deciding the ability of individual animals to succumb to or resist the effects of a given chemical/disease.

*How efficient are differential expression techniques at recovering a high percentage of differentially expressed genes?*

A number of groups have produced experimental data suggesting that mammalian cells produce between 8000–15000 different mRNA species at any one time (Mechler and Rabbitts 1981, Hedrick *et al.* 1984, Bravo 1990), although figures as high as 20–30000 have also been quoted (Axel *et al.* 1976). Hedrick *et al.* (1984) provided evidence suggesting that the majority of these belong to the rare abundance class. A breakdown of this abundance distribution is shown in table 1.

When the results of differential display experiments have been compared with data obtained previously using other methods, it is apparent that not all differentially expressed mRNAs are represented in the final display. In particular, rare messages (which, importantly, often include regulatory proteins) are not easily recovered using differential display systems. This is a major shortcoming, as the majority of mRNA species exist at levels of less than 0.005 % of the total population (table 1). Bertoli *et al.* (1995) examined the efficiency of DD templates (heterogeneous mRNA populations) for recovering rare messages and were unable to detect mRNA

species present at less than 1.2% of the total mRNA population—equivalent to an intermediate or abundant species. Interestingly, when simple model systems (single target only) were used instead of a heterogeneous mRNA population, the same primers could detect levels of target mRNA down to 10000 × smaller. These results are probably best explained by competition for substrates from the many PCR products produced in a DD reaction.

The numbers of differentially expressed mRNAs reported in the literature using various model systems provides further evidence that many differentially expressed mRNAs are not recovered. For example, DeRisi *et al.* (1997) used DNA array technology to examine gene expression in yeast following exhaustion of sugar in the medium, and found that more than 1700 genes showed a change in expression of at least 2-fold. In light of such a finding, it would not be unreasonable to suggest that of the 8000–15 000 different mRNA species produced by any given mammalian cell, up to 1000 or more may show altered expression following chemical stimulation. Whilst this may be an extreme figure, it is known that at least 100 genes are activated/upregulated in Jurkat (T-) cells following IL-2 stimulation (Ullman *et al.* 1990). In addition, Wan *et al.* (1996) estimated that interferon- $\gamma$ -stimulated HeLa cells differentially express up to 433 genes (assuming 24000 distinct mRNAs expressed by the cells). However, there have been few publications documenting anywhere near the recovery of these numbers. For example, in using DD to compare normal and regenerating mouse liver, Bauer *et al.* (1993) found only 70 of 38000 total bands to be different. Of these, 50% (35 genes) were shown to correspond to differentially expressed bands. Chen *et al.* (1996) reported 10 genes upregulated in female rat liver following ethinyl estradiol treatment. McKenzie and Drake (1997) identified 14 different gene products whose expression was altered by phorbol myristate acetate (PMA, a tumour promoter agent) stimulation of a human myelomonocytic cell line. Kilty and Vickers (1997) identified 10 different gene products whose expression was upregulated in the peripheral blood leukocytes of allergic disease sufferers. Linskens *et al.* (1995) found 23 genes differentially expressed between young and senescent fibroblasts. Techniques other than DD have also provided an apparent paucity of differentially expressed genes. Using SH for example, Cao *et al.* (1997) found 15 genes differentially expressed in colorectal cancer compared to normal mucosal epithelium. Fitzpatrick *et al.* (1995) isolated 17 genes upregulated in rat liver following treatment with the peroxisome proliferator, clofibrate; Philips *et al.* (1990) isolated 12 cDNA clones which were upregulated in highly metastatic mammary adenocarcinoma cell lines compared to poorly metastatic ones. Prashar and Weissman (1996) used 3' restriction fragment analysis and identified approximately 40 genes showing altered expression within 4 h of activation of Jurkat T-cells. Groenink and Leegwater (1996) analysed 27 gene fragments isolated using SSH of delayed early response phase of liver regeneration and found only 12 to be upregulated.

In the laboratory, SSH was used to isolate up to 70 candidate genes which appear to show altered expression in guinea pig liver following short-term treatment with the peroxisome proliferator, WY-14,643 (Rockets, Swales, Esdaile and Gibson, unpublished observations). However, these findings have still to be confirmed by analysis of the extracted tissue mRNA for differential expression of these sequences. Whilst the latest differential display technologies are purported to include design and experimental modifications to overcome this lack of efficiency (in both the total number of differentially expressed genes recovered and the percentage that are true

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positives), it is still not clear if such adaptations are practically effective—proving efficiency by spiking with a known amount of limited numbers of artificial construct(s) is one thing, but isolating a high percentage of the rare messages already present in an mRNA population is another. Of course, some models will genuinely produce only a small number of differentially expressed genes. In addition, there are also technical problems that can reduce efficiency. For example, mRNAs may have an unusual primary structure that effectively prevents their amplification by PCR-based systems. In addition, it is known that under certain circumstances not all mRNAs have 3' polyA sites. For example, during *Xenopus* development, deadenylation is used as a means to stabilize RNAs (Voeltz and Steitz 1998), whilst preferential deadenylation may play a role in regulating Hsp70 (and perhaps, therefore, other stress protein) expression in *Drosophila* (Dellavalle et al. 1994). The presence of deadenylated mRNAs would clearly reduce the efficiency of systems utilizing a polydT reverse transcription step. The efficiency of any system also depends on the quality of the starting material. All differential display techniques use mRNA as their target material. However, it is difficult to isolate mRNA that is completely free of ribosomal RNA. Even if polydT primers are used to prime first strand cDNA synthesis, ribosomal RNA is often transcribed to some degree (Clontech PCR-Select cDNA Subtraction kit user manual). It has been shown, at least in the case of SSH, that a high rRNA:mRNA ratio can lead to inefficient subtractive hybridization (Clontech PCR-Select cDNA Subtraction kit user manual), and there is no reason to suppose that it will not do likewise in other SH approaches. Finally, those techniques that utilise a presubtraction amplification step (e.g. RDA) may present a skewed representation since some sequences amplify better than others.

Of course, probably the most important consideration is the temporal factor. It is clear that any given differential display experiment can only interrogate a cell at one point in time. It may well be that a high percentage of the genes showing altered expression at that time are obtained. However, given that disease processes and responses to environmental stimuli involve dynamic cascades of signalling, regulation, production and action, it is clear that all those genes which are switched on/off at different times will not be recovered and, therefore, vital information may well be missed. It is, therefore, imperative to obtain as much information about the model system beforehand as possible, from which a strategy can be derived for targeting specific time points or events that are of particular interest to the investigator. One way of getting round this problem of single time point analysis is to conduct the experiment over a suitable time course which, of course, adds substantially to the amount of work involved.

#### *How sensitive are differential expression technologies?*

There has been little published data that addresses the issue of how large the change in expression must be for it to permit isolation of the gene in question with the various differential expression technologies. Although the isolation of genes whose expression is changed as little as 1.5-fold has been reported using SSH (Groenink and Leegwater-1996), it appears that those demonstrating a change in excess of 5-fold are more likely to be picked up. Thus, there is a 'grey zone' in between where small changes could fade in and out of isolation between

experiments and animals. DD, on the other hand, is not subject to this grey zone since, unlike SH approaches, it does not amplify the difference in expression between two samples. Wan *et al.* (1996) reported that differences in expression of twofold or more are detectable using DD.

#### *Resolution and visualization of differential expression products*

It seems highly improbable with current technology that a gel system could be developed that is able to resolve all gene species showing altered expression in any given test system (be it SH- or DD-based). Polyacrylamide gel electrophoresis (PAGE) can resolve size differences down to 0.2% (Sambrook *et al.* 1989) and are used as standard in DD experiments. Even so, it is clear that a complex series of gene products such as those seen in a DD will contain unresolvable components. Thus, what appears to be one band in a gel may in fact turn out to be several. Indeed, it has been well documented (Mathieu-Daude *et al.* 1996, Smith *et al.* 1997) that a single band extracted from a DD often represents a composite of heterogeneous products, and the same has been found for SSH displays in this laboratory (Rockett *et al.* 1997). One possible solution was offered by Mathieu-Daude *et al.* (1996), who extracted and reamplified candidate bands from a DD display and used single strand conformation polymorphism (SSCP) analysis to confirm which components represented the truly differentially expressed product.

Many scientists often try to avoid the use of PAGE where possible because it is technically more demanding than agarose gel electrophoresis (AGE). Unfortunately, high resolution agarose gels such as Metaphor (FMC, Lichfield, UK) and AquaPor HR (National Diagnostics, Hesse, UK), whilst easier to prepare and manipulate than PAGE, can only separate DNA sequences which differ in size by around 1.5–2% (15–20 base pairs for a 1Kb fragment). Thus, SSH, RDA or other such products which differ in size by less than this amount are normally not resolvable. However, a simple technique does in fact exist for increasing the resolving power of AGE—the inclusion of HA-red (10-phenyl neutral red-PEG ligand) or HA-yellow (bisbenzamide-PEG ligand) (Hanse Analytik GmbH, Bremen, Germany) in a gel separates identical or closely sized products on base content. Specifically, HA-red and -yellow selectively bind to GC and AT DNA motifs, respectively (Wawer *et al.* 1995, Hanse Analytik 1997, personal communication). Since both HA-stains possess an overall positive charge, they migrate towards the cathode when an electric field is applied. This is in direct opposition to DNA, which is negatively charged and, therefore, migrates towards the anode. Thus, if two DNA clones are identical in size (as perceived on a standard high resolution agarose gel), but differ in AT/GC content, inclusion of a HA-dye in the gel will effectively retard the migration of one of the sequences compared to the other, effectively making it apparently larger and, thus, providing a means of differentiating between the two. The use of HA-red has been shown to resolve sequences with an AT variation of less than 1% (Wawer *et al.* 1995), whilst Hanse Analytik have reported that HA staining is so sensitive that in one case it was used to distinguish two 567bp sequences which differed by only a single point mutation (Hanse Analytik 1996, personal communication). Therefore, if one wishes to check whether all the clones produced from a specific band in a differential display experiment are derived from the same gene species, a small amount of reamplified or digested clone can be run on a standard high resolution gel, and a second aliquot

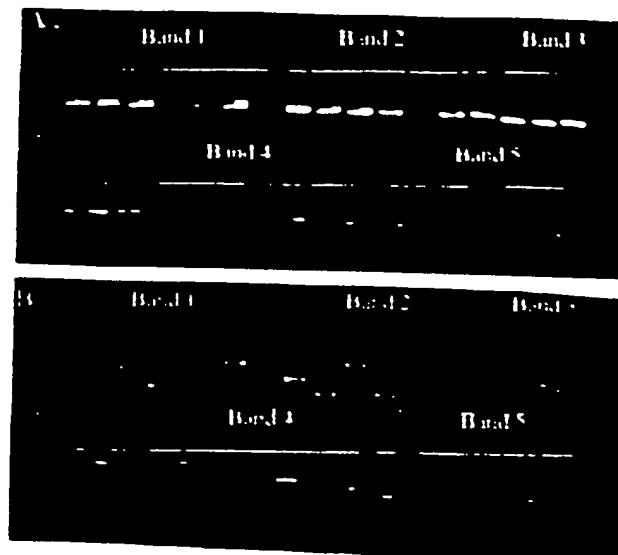


Figure 10. Discrimination of clones of identical/nearly identical size using HA-red. Bands of decreasing size (1–5) were extracted from the final display of a suppression subtractive hybridization experiment and cloned. Seven colonies were picked at random from each cloned band and their inserts amplified using PCR. The products were run on two gels, (A) a high resolution 2% agarose gel, and (B) a high resolution 2% agarose gel containing 1 U/ml HA-red. With few exceptions, all the clones from each band appear to be the same size (gel A). However, the presence of HA-red (gel B), which separates identically-sized DNA fragments based on the percentage of GC within the sequence, clearly indicates the presence of different gene species within each band. For example, even though all five re-amplified clones of band 1 appear to be the same size, at least four different gene species are represented.

in a similar gel containing one of the HA-stains. The standard gel should indicate any gross size differences, whilst the HA-stained gel should separate otherwise unresolvable species (on standard AGE) according to their base content. Geisinger *et al.* (1997) reported successful use of this approach for identifying DD-derived clones. Figure 10 shows such an experiment carried out in this laboratory on clones obtained from a band extracted from an SSH display.

An alternative approach is to carry out a 2-D analysis of the differential display products. In this approach, size-based separation is first carried out in a standard agarose gel. The gel slice containing the display is then extracted and incorporated in to a HA gel for resolution based on AT/GC content.

Of course, one should always consider the possibility of there being different gene species which are the same size and have the same GC/AT content. However, even these species are not unresolvable given some effort—again, one might use SSCP, or perhaps a denaturing gradient gel electrophoresis (DGGE) or temperature gradient field electrophoresis (TGGE) approach to resolve the contents of a band, either directly on the extracted band (Suzuki *et al.* 1991) or on the reamplified product.

The requirement of some differential display techniques to visualize large numbers of products (e.g. DD and GEF) can also present a problem in that, in terms of numbers, the resolution of PAGE rarely exceeds 300–400 bands. One approach to overcoming this might be to use 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991).

Extraction of differentially expressed bands from a gel can be complex since, in some cases (e.g. DD, GEF), the results are visualized by autoradiographic means, such that precise overlay of the developed film on the gel must occur if the correct band is to be extracted for further analysis. Clearly, a misjudged extraction can account for many man-hours lost. This problem, and that of the use of radioisotopes, has been addressed by several groups. For example, Lohmann *et al.* (1995) demonstrated that silver staining can be used directly to visualize DD bands in horizontal PAGs. An *et al.* (1996) avoided the use of radioisotopes by transferring a small amount (20–30%) of the DNA from their DD to a nylon membrane, and visualizing the bands using chemiluminescent staining before going back to extract the remaining DNA from the gel. Chen and Peck (1996) went one step further and transferred the entire DD to a nylon membrane. The DNA bands were then visualized using a digoxigenin (DIG) system (DIG was attached to the polydT primers used in the differential display procedure). Differentially expressed bands were cut from the membrane and the DNA eluted by washing with PCR buffer prior to reamplification.

One of the advantages of using techniques such as SSH and RDA is that the final display can be run on an agarose gel and the bands visualized with simple ethidium bromide staining. Whilst this approach can provide acceptable results, over staining with SYBR Green I or SYBR Gold nucleic acid stains (FMC) effectively enhances the intensity and sharpness of the bands. This greatly aids in their precise extraction and often reveals some faint products that may otherwise be overlooked. Whilst differential displays stained with SYBR Green I are better visualized using short wavelength UV (254 nm) rather than medium wavelength (306 nm), the shorter wavelength is much more DNA damaging. In practice, it takes only a few seconds to damage DNA extracted under 254 nm irradiation, effectively preventing reamplification and cloning. The best approach is to over stain with SYBR Green I and extract bands under a medium wavelength UV transillumination.

### The possible use of 'microfingerprinting' to reduce complexity

Given the sheer number of gene products and the possible complexity of each band, an alternative approach to rapid characterization may be to use an enhanced analysis of a small section of a differential display—a 'sub-fingerprint' or 'micro-fingerprint'. In this case, one could concentrate on those bands which only appear in a particular chosen size region. Reducing the fingerprint in this way has at least two advantages. One is that it should be possible to use different gel types, concentrations and run times tailored exactly to that region. Currently, one might run products from 100–3000+ bp on the same gel, which leads to compromise in the gel system being used and consequently to suboptimal resolution, both in terms of size and numbers, and can lead to problems in the accurate excision of individual bands. Secondly, it may be possible to enhance resolution by using a 2-D analysis using a HA-stain, as described earlier. In summary, if a range of gene product sizes is carefully chosen to include certain 'relevant' genes, the 2-D system standardized, and appropriate gene analysis used, it may be possible to develop a method for the early and rapid identification of compounds which have similar or widely different cellular effects. If the prognosis for exposure to one or more other chemicals which display a similar profile is already known, then one could perhaps predict similar effects for any new compounds which show a similar micro-fingerprint.

An alternative approach to microfingerprinting is to examine altered expression in specific families of genes through careful selection of PCR primers and/or post-reaction analysis. Stress genes, growth factors and/or their receptors, cell cycling genes, cytochromes P450 and regulatory proteins might be considered as candidates for analysis in this way. Indeed, some off-the-shelf DNA arrays (e.g. Clontech's Atlas cDNA Expression Array series) already anticipated this to some degree by grouping together genes involved in different responses e.g. apoptosis, stress, DNA-damage response etc.

## Screening

### *False positives*

The generation of false positives has been discussed at length amongst the differential display community (Liang *et al.* 1993, 1995, Nishio *et al.* 1994, Sun *et al.* 1994, Sompayrac *et al.* 1995). The reason for false positives varies with the technique being used. For instance, in RDA, the use of adaptors which have not been HPLC purified can lead to the production of false positives through illegitimate ligation events (O'Neill and Sinclair 1997), whilst in DD they can arise through PCR artifacts and illegitimate transcription of rRNA. In SH, false positives appear to be derived largely from abundant gene species, although some may arise from cDNA/mRNA species which do not undergo hybridization for technical reasons.

A quick screening of putative differentially expressed clones can be carried out using a simple dot blot approach, in which labelled first strand probes synthesized from tester and driver mRNA are hybridized to an array of said clones (Hedrick *et al.* 1984, Sakaguchi *et al.* 1986). Differentially expressed clones will hybridize to tester probe, but not driver. The disadvantage of this approach is that rare species may not generate detectable hybridization signals. One option for those using SSH is to screen the clones using a labelled probe generated from the subtracted cDNA from which it was derived, and with a probe made from the reverse subtraction reaction (ClonTechniques 1997a). Since the SSH method enriches rare sequences, it should be possible to confirm the presence of clones representing low abundance genes. Despite this quick screening step, there is still the need to go back to the original mRNA and confirm the altered expression using a more quantitative approach. Although this may be achieved using Northern blots, the sensitivity is poor by today's high standards and one must rely on PCR methods for accurate and sensitive determinations (see below).

## Sequence analysis

The majority of differential display procedures produce final products which are between 100 and 1000bp in size. However, this may considerably reduce the size of the sequence for analysis of the DNA databases. This in turn leads to a reduced confidence in the result—several families of genes have members whose DNA sequences are almost identical except in a few key stretches, e.g. the cytochrome P450 gene superfamily (Nelson *et al.* 1996). Thus, does the clone identified as being almost identical to gene  $X_0$  really come from that gene, or its brother gene  $X_1$  or its as yet undiscovered sister  $X_2$ ? For example, using SSH, part of a gene was isolated,

mine altered expression R primers and/or post-receptors, cell cycling considered as candidates arrays (e.g. Clontech's this to some degree by apoptosis, stress, DNA-

which was up-regulated in the liver of rats exposed to Wy-14,643 and was identified by a FASTA search as being transferrin (data not shown). However, transferrin is known to be downregulated by hypolipidemic peroxisome proliferators such as Wy-14,643 (Hertz *et al.* 1996), and this was confirmed with subsequent RT-PCR analysis. This suggests that the gene sequence isolated may belong to a gene which is closely related to transferrin, but is regulated by a different mechanism.

A further problem associated with SH technology is redundancy. In most cases before SH is carried out, the cDNA population must first be simplified by restriction digestion. This is important for at least two reasons:

- (1) To reduce complexity—long cDNA fragments may form complex networks which prevent the formation of appropriate hybrids, especially at the high concentrations required for efficient hybridization.
- (2) Cutting the cDNAs into small fragments provides better representation of individual genes. This is because genes derived from related but distinct members of gene families often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (Ko 1990). Furthermore, different fragments from the same cDNA may differ considerably in terms of hybridization and amplification and, thus, may not efficiently do one or the other (Wang and Brown 1991). Thus, some fragments from differentially expressed cDNAs may be eliminated during subtractive hybridization procedures. However, other fragments may be enriched and isolated. As a consequence of this, some genes will be cut one or more times, giving rise to two or more fragments of different sizes. If those same genes are differentially expressed, then two or more of the different size fragments may come through as separate bands on the final differential display, increasing the observed redundancy and increasing the number of redundant sequencing reactions.

Sequence comparisons also throw up another important point—at what degree of sequence similarity does one accept a result. Is 90% identity between a gene derived from your model species and another acceptably close? Is 95% between your sequence and one from the same species also acceptable? This problem is particularly relevant when the forward and reverse sequence comparisons give similar sequences with completely different gene species! An arbitrary decision seems to be to allocate genes that are definite (95% and above similarity) and then group those between 60 and 95% as being related or possible homologues.

### Quantitative analysis

At some point, one must give consideration to the quantitative analysis of the candidate genes, either as a means of confirming that they are truly differentially expressed, or in order to establish just what the differences are. Northern blot analysis is a popular approach as it is relatively easy and quick to perform. However, the major drawback with Northern blots is that they are often not sensitive enough to detect rare sequences. Since the majority of messages expressed in a cell are of low abundance (see table 1), this is a major problem. Consequently, RT-PCR may be the method of choice for confirming differential expression. Although the procedure is somewhat more complex than Northern analysis, requiring synthesis of primers and optimization of reaction conditions for each gene species, it is now possible to set up high throughput PCR systems using multichannel pipettes, 96+-well plates and

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appropriate thermal cycling technology. Whilst quantitative analysis is more desirable, being more accurate and without reliance on an internal standard, the money and time needed to develop a competitor molecule is often excessive, especially when one might be examining tens or even hundreds of gene species. The use of semi-quantitative analysis is simpler, although still relatively involved. One must first of all choose an internal standard that does not change in the test cells compared to the controls. Numerous reference genes have been tried in the past, for example interferon-gamma (IFN- $\gamma$ , Frye *et al.* 1989),  $\beta$ -actin (Heuval *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Wong *et al.* 1994), dihydrofolate reductase (DHFR, Mohler and Butler 1991),  $\beta$ -2-microglobulin ( $\beta$ -2-m, Murphy *et al.* 1990), hypoxanthine phosphoribosyl transferase (HPRT, Foss *et al.* 1998) and a number of others (ClonTechniques 1997b). Ideally, an internal standard should not change its level of expression in the cell regardless of cell age, stage in the cell cycle or through the effects of external stimuli. However, it has been shown on numerous occasions that the levels of most housekeeping genes currently used by the research community do in fact change under certain conditions and in different tissues (ClonTechniques 1997b). It is imperative, therefore, that preliminary experiments be carried out on a panel of housekeeping genes to establish their suitability for use in the model system.

Interpretation of quantitative data must also be treated with caution. By comparing the lists of genes identified by differential expression one can perhaps gain insight into why two different species react in different ways to external stimuli. For example, rats and mice appear sensitive to the non-genotoxic effects of a wide range of peroxisome proliferators whilst Syrian hamsters and guinea pigs are largely resistant (Orton *et al.* 1984, Rodricks and Turnbull 1987, Lake *et al.* 1989, 1993, Makowska *et al.* 1992). A simplified approach to resolving the reason(s) why is to compare lists of up- and down-regulated genes in order to identify those which are expressed in only one species and, through background knowledge of the effects of the said gene, might suggest a mechanism of facilitated non-genotoxic carcinogenesis or protection. Of course, the situation is likely to be far more complex. Perhaps if there were one key gene protecting guinea pig from non-genotoxic effects and it was upregulated 50 times by PPs, the same gene might only be up-regulated five times in the rat. However, since both were noted to be upregulated, the importance of the gene may be overlooked. Just to complicate matters, a large change in expression does not necessarily mean a biologically important change. For example, what is the true relevance of gene Y which shows a 50-fold increase after a particular treatment, and gene Z which shows only a 5-fold increase? If one examines the literature one may find that historically, gene Y has often been shown to be up-regulated 40–60-fold by a number of unrelated stimuli—in light of this the 50-fold increase would appear less significant. However, the literature may show that gene Z has never been recorded as having more than doubled in expression—which makes your 5-fold increase all the more exciting. Perhaps even more interesting is if that same 5-fold increase has only been seen in related neoplasms or following treatment with related chemicals.

#### Problems in using the differential display approach

Differential display technology originally held promise of an easily obtainable 'fingerprint' of those genes which are up- or down-regulated in test animals/cells in a developmental process or following exposure to given stimuli. However, it has

ative analysis is more internal standard, the rule is often excessive, eds of gene species. The relatively involved. One change in the test cells been tried in the past, for in (Heuval *et al.* 1994), Vong *et al.* 1994); di- $\beta$ -2-microglobulin ( $\beta$ -2-sferase (HPRT, Foss *et al.* b). Ideally, an internal ll regardless of cell age, li. However, it has been keeping genes currently ertain conditions and in e, therefore, that pre-eping genes to establish

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become clear that the fingerprinting process, whilst still valid, is much too complex to be represented by a single technique profile. This is because all differential display techniques have common and/or unique technical problems which preclude the isolation and identification of all those genes which show changes in expression. Furthermore, there are important genetic changes related to disease development which differential expression analysis is simply not designed to address. An example of this is the presence of small deletions, insertions, or point mutations such as those seen in activated oncogenes, tumour suppressor genes and individual polymorphisms. Polymorphic variations, small though they usually are, are often regarded as being of paramount importance in explaining why some patients respond better than others to certain drug treatments (and, in logical extension, why some people are less affected by potentially dangerous xenobiotics/carcinogens than others). The identification of such point mutations and naturally occurring polymorphisms requires the subsequent application of sequencing, SSCP, DGGE or TGGE to the gene of interest. Furthermore, differential display is not designed to address issues such as alternatively spliced gene species or whether an increased abundance of mRNA is a result of increased transcription or increased mRNA stability.

### Conclusions

Perhaps the main advantage of open system differential display techniques is that they are not limited by extant theories or researcher bias in revealing genes which are differentially expressed, since they are designed to amplify all genes which demonstrate altered expression. This means that they are useful for the isolation of previously unknown genes which may turn out be useful biomarkers of a particular state or condition. At least one open system (SAGE) is also quantitative, thus eliminating the need to return to the original mRNA and carry out Northern/PCR analysis to confirm the result. However, the rapid progress of genome mapping projects means that over the next 5–10 years or so, the balance of experimental use will switch from open to closed differential display systems, particularly DNA arrays. Arrays are easier and faster to prepare and use, provide quantitative data, are suitable for high throughput analysis and can be tailored to look at specific signalling pathways or families of genes. Identification of all the gene sequences in human and common laboratory animals combined with improved DNA array technology, means that it will soon no longer be necessary to try to isolate differentially expressed genes using the technically more demanding open system approach. Thus, their main advantage (that of identifying unknown genes) will be largely eradicated. It is likely, therefore, that their sphere of application will be reduced to analysis of the less common laboratory species, since it will be some time yet before the genomes of such animals as zebrafish, electric eels, gerbils, crayfish and squid, for example, will be sequenced.

Of course, in the end the question will always remain: What is the functional/biological significance of the identified, differentially expressed genes? One persistent problem is understanding whether differentially expressed genes are a cause or consequence of the altered state. Furthermore, many chemicals, such as non-genotoxic carcinogens, are also mitogens and so genes associated with replication will also be upregulated but may have little or nothing to do with the

carcinogenic effect. Whilst differential display technology cannot hope to answer these questions, it does provide a springboard from which identification, regulatory and functional studies can be launched. Understanding the molecular mechanism of cellular responses is almost impossible without knowing the regulation and function of those genes and their condition (e.g. mutated). In an abstract sense, differential display can be likened to a still photograph, showing details of a fixed moment in time. Consider the Historian who knows the outcome of a battle and the placement and condition of the troops before the battle commenced, but is asked to try and deduce how the battle progressed and why it ended as it did from a few still photographs—an impossible task. In order to understand the battle, the Historian must find out the capabilities and motivation of the soldiers and their commanding officers, what the orders were and whether they were obeyed. He must examine the terrain, the remains of the battle and consider the effects the prevailing weather conditions exerted. Likewise, if mechanistic answers are to be forthcoming, the scientist must use differential display in combination with other techniques, such as knockout technology, the analysis of cell signalling pathways, mutation analysis and time and dose response analyses. Although this review has emphasized the importance of differential gene profiling, it should not be considered in isolation and the full impact of this approach will be strengthened if used in combination with functional genomics and proteomics (2-dimensional protein gels from isoelectric focusing and subsequent SDS electrophoresis and virtual 2D-maps using capillary electrophoresis). Proteomics is attracting much recent attention as many of the changes resulting in differential gene expression do not involve changes in mRNA levels, as described extensively herein, but rather protein-protein, protein-DNA and protein phosphorylation events which would require functional genomics or proteomic technologies for investigation.

Despite the limitations of differential display technology, it is clear that many potential applications and benefits can be obtained from characterizing the genetic changes that occur in a cell during normal and disease development and in response to chemical or biological insult. In light of functional data, such profiling will provide a 'fingerprint' of each stage of development or response, and in the long term should help in the elucidation of specific and sensitive biomarkers for different types of chemical/biological exposure and disease states. The potential medical and therapeutic benefits of understanding such molecular changes are almost immeasurable. Amongst other things, such fingerprints could indicate the family or even specific type of chemical an individual has been exposed to plus the length and/or acuteness of that exposure, thus indicating the most prudent treatment. They may also help uncover differences in histologically identical cancers, provide diagnostic tests for the earliest stages of neoplasia and, again, perhaps indicate the most efficacious treatment.

The Human Genome Project will be completed early in the next century and the DNA sequence of all the human genes will be known. The continuing development and evolution of differential gene expression technology will ensure that this knowledge contributes fully to the understanding of human disease processes.

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## Assessing sequence comparison methods with reliable structurally identified distant evolutionary relationships

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**ABSTRACT** Pairwise sequence comparison methods have been assessed using proteins whose relationships are known reliably from their structures and functions, as described in the SCOP database [Murzin, A. G., Brenner, S. E., Hubbard, T. & Chothia C. (1995) *J. Mol. Biol.* 247, 536–540]. The evaluation tested the programs BLAST [Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410], WU-BLAST2 [Altschul, S. F. & Gish, W. (1996) *Methods Enzymol.* 266, 460–480], FASTA [Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 2444–2448], and SSEARCH [Smith, T. F. & Waterman, M. S. (1981) *J. Mol. Biol.* 147, 195–197] and their scoring schemes. The error rate of all algorithms is greatly reduced by using statistical scores to evaluate matches rather than percentage identity or raw scores. The E-value statistical scores of SSEARCH and FASTA are reliable: the number of false positives found in our tests agrees well with the scores reported. However, the P-values reported by BLAST and WU-BLAST2 exaggerate significance by orders of magnitude. SSEARCH, FASTA k<sub>tup</sub> = 1, and WU-BLAST2 perform best, and they are capable of detecting almost all relationships between proteins whose sequence identities are >30%. For more distantly related proteins, they do much less well; only one-half of the relationships between proteins with 20–30% identity are found. Because many homologs have low sequence similarity, most distant relationships cannot be detected by any pairwise comparison method; however, those which are identified may be used with confidence.

Sequence database searching plays a role in virtually every branch of molecular biology and is crucial for interpreting the sequences issuing forth from genome projects. Given the method's central role, it is surprising that overall and relative capabilities of different procedures are largely unknown. It is difficult to verify algorithms on sample data because this requires large data sets of proteins whose evolutionary relationships are known unambiguously and independently of the methods being evaluated. However, nearly all known homologs have been identified by sequence analysis (the method to be tested). Also, it is generally very difficult to know, in the absence of structural data, whether two proteins that lack clear sequence similarity are unrelated. This has meant that although previous evaluations have helped improve sequence comparison, they have suffered from insufficient, imperfectly characterized, or artificial test data. Assessment also has been problematic because high quality database sequence searching attempts to have both sensitivity (detection of homologs) and specificity (rejection of unrelated proteins); however, these complementary goals are linked such that increasing one causes the other to be reduced.

Sequence comparison methodologies have evolved rapidly, so no previously published tests have evaluated modern versions of programs commonly used. For example, parameters in BLAST (1) have changed, and WU-BLAST2 (2)—which produces gapped alignments—has become available. The latest version of FASTA (3) previously tested was 1.6, but the current release (version 3.0) provides fundamentally different results in the form of statistical scoring.

The previous reports also have left gaps in our knowledge. For example, there has been no published assessment of thresholds for scoring schemes more sophisticated than percentage identity. Thus, the widely discussed statistical scoring measures have never actually been evaluated on large databases of real proteins. Moreover, the different scoring schemes commonly in use have not been compared.

Beyond these issues, there is a more fundamental question: in an absolute sense, how well does pairwise sequence comparison work? That is, what fraction of homologous proteins can be detected using modern database searching methods?

In this work, we attempt to answer these questions and to overcome both of the fundamental difficulties that have hindered assessment of sequence comparison methodologies. First, we use the set of distant evolutionary relationships in the SCOP: Structural Classification of Proteins database (4), which is derived from structural and functional characteristics (5). The SCOP database provides a uniquely reliable set of homologs, which are known independently of sequence comparison. Second, we use an assessment method that jointly measures both sensitivity and specificity. This method allows straightforward comparison of different sequence searching procedures. Further, it can be used to aid interpretation of real database searches and thus provide optimal and reliable results.

**Previous Assessments of Sequence Comparison.** Several previous studies have examined the relative performance of different sequence comparison methods. The most encompassing analyses have been by Pearson (6, 7), who compared the three most commonly used programs. Of these, the Smith–Waterman algorithm (8) implemented in SSEARCH (3) is the oldest and slowest but the most rigorous. Modern heuristics have provided BLAST (1) the speed and convenience to make it the most popular program. Intermediate between these two is FASTA (3), which may be run in two modes offering either greater speed (k<sub>tup</sub> = 2) or greater effectiveness (k<sub>tup</sub> = 1). Pearson also considered different parameters for each of these programs.

To test the methods, Pearson selected two representative proteins from each of 67 protein superfamilies defined by the PIR database (9). Each was used as a query to search the database, and the matched proteins were marked as being homologous or unrelated according to their membership of PIR

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Abbreviation: EPQ, errors per query.

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superfamilies. Pearson found that modern matrices and "ln-scaling" of raw scores improve results considerably. He also reported that the rigorous Smith-Waterman algorithm worked slightly better than FASTA, which was in turn more effective than BLAST.

Very large scale analyses of matrices have been performed (10), and Henikoff and Henikoff (11) also evaluated the effectiveness of BLAST and FASTA. Their test with BLAST considered the ability to detect homologs above a predetermined score but had no penalty for methods which also reported large numbers of spurious matches. The Henikoffs searched the SWISS-PROT database (12) and used PROSITE (13) to define homologous families. Their results showed that the BLOSUM62 matrix (14) performed markedly better than the extrapolated PAM-series matrices (15), which previously had been popular.

A crucial aspect of any assessment is the data that are used to test the ability of the program to find homologs. But in Pearson's and the Henikoffs' evaluations of sequence comparison, the correct results were effectively unknown. This is because the superfamilies in PIR and PROSITE are principally created by using the same sequence comparison methods which are being evaluated. Interdependency of data and methods creates a "chicken and egg" problem, and means for example, that new methods would be penalized for correctly identifying homologs missed by older programs. For instance, immunoglobulin variable and constant domains are clearly homologous, but PIR places them in different superfamilies. The problem is widespread: each superfamily in PIR 48.00 with a structural homolog is itself homologous to an average of 1.6 other PIR superfamilies (16).

To surmount these sorts of difficulties, Sander and Schneider (17) used protein structures to evaluate sequence comparison. Rather than comparing different sequence comparison algorithms, their work focused on determining a length-dependent threshold of percentage identity, above which all proteins would be of similar structure. A result of this analysis was the HSP equation; it states that proteins with 25% identity over 80 residues will have similar structures, whereas shorter alignments require higher identity. (Other studies also have used structures (18–20), but these focused on a small number of model proteins and were principally oriented toward evaluating alignment accuracy rather than homology detection.)

A general solution to the problem of scoring comes from statistical measures (i.e., E-values and P-values) based on the extreme value distribution (21). Extreme value scoring was implemented analytically in the BLAST program using the Karlin and Altschul statistics (22, 23) and empirical approaches have been recently added to FASTA and SSEARCH. In addition to being heralded as a reliable means of recognizing significantly similar proteins (24, 25), the mathematical tractability of statistical scores "is a crucial feature of the BLAST algorithm" (1). The validity of this scoring procedure has been tested analytically and empirically (see ref. 2 and references in ref. 24). However, all large empirical tests used random sequences that may lack the subtle structure found within biological sequences (26, 27) and obviously do not contain any real homologs. Thus, although many researchers have suggested that statistical scores be used to rank matches (24, 25, 28), there have been no large rigorous experiments on biological data to determine the degree to which such rankings are superior.

**A Database for Testing Homology Detection.** Since the discovery that the structures of hemoglobin and myoglobin are very similar though their sequences are not (29), it has been apparent that comparing structures is a more powerful (if less convenient) way to recognize distant evolutionary relationships than comparing sequences. If two proteins show a high degree of similarity in their structural details and function, it

is very probable that they have an evolutionary relationship though their sequence similarity may be low.

The recent growth of protein structure information combined with the comprehensive evolutionary classification in the SCOP database (4, 5) have allowed us to overcome previous limitations. With these data, we can evaluate the performance of sequence comparison methods on real protein sequences whose relationships are known confidently. The SCOP database uses structural information to recognize distant homologs, the large majority of which can be determined unambiguously. These superfamilies, such as the globins or the immunoglobulins, would be recognized as related by the vast majority of the biological community despite the lack of high sequence similarity.

From SCOP, we extracted the sequences of domains of proteins in the Protein Data Bank (PDB) (30) and created two databases. One (PDB90D-B) has domains, which were all <90% identical to any other, whereas (PDB40D-B) had those <40% identical. The databases were created by first sorting all protein domains in SCOP by their quality and making a list. The highest quality domain was selected for inclusion in the database and removed from the list. Also removed from the list (and discarded) were all other domains above the threshold level of identity to the selected domain. This process was repeated until the list was empty. The PDB40D-B database contains 1,323 domains, which have 9,044 ordered pairs of distant relationships, or  $\approx 0.5\%$  of the total 1,749,006 ordered pairs. In PDB90D-B, the 2,079 domains have 53,988 relationships, representing 1.2% of all pairs. Low complexity regions of sequence can achieve spurious high scores, so these were masked in both databases by processing with the SEG program (27) using recommended parameters: 12 1.8 2.0. The databases used in this paper are available from <http://sss.stanford.edu/sss/>, and databases derived from the current version of SCOP may be found at <http://scop.mrc-lmb.cam.ac.uk/scop/>.

Analyses from both databases were generally consistent, but PDB40D-B focuses on distantly related proteins and reduces the heavy overrepresentation in the PDB of a small number of families (31, 32), whereas PDB90D-B (with more sequences) improves evaluations of statistics. Except where noted otherwise, the distant homolog results here are from PDB40D-B. Although the precise numbers reported here are specific to the structural domain databases used, we expect the trends to be general.

**Assessment Data and Procedure.** Our assessment of sequence comparison may be divided into four different major categories of tests. First, using just a single sequence comparison algorithm at a time, we evaluated the effectiveness of different scoring schemes. Second, we assessed the reliability of scoring procedures, including an evaluation of the validity of statistical scoring. Third, we compared sequence comparison algorithms (using the optimal scoring scheme) to determine their relative performance. Fourth, we examined the distribution of homologs and considered the power of pairwise sequence comparison to recognize them. All of the analyses used the databases of structurally identified homologs and a new assessment criterion.

The analyses tested BLAST (1), version 1.4.9MP, and WU-BLAST2 (2), version 2.0a13MP. Also assessed was the FASTA package, version 3.0t76 (3), which provided FASTA and the SSEARCH implementation of Smith-Waterman (8). For SSEARCH and FASTA, we used BLOSUM45 with gap penalties  $-12/-1$  (7, 16). The default parameters and matrix (BLOSUM62) were used for BLAST and WU-BLAST2.

**The "Coverage Vs. Error" Plot.** To test a particular protocol (comprising a program and scoring scheme), each sequence from the database was used as a query to search the database. This yielded ordered pairs of query and target sequences with associated scores, which were sorted, on the basis of their scores, from best to worst. The ideal method would have

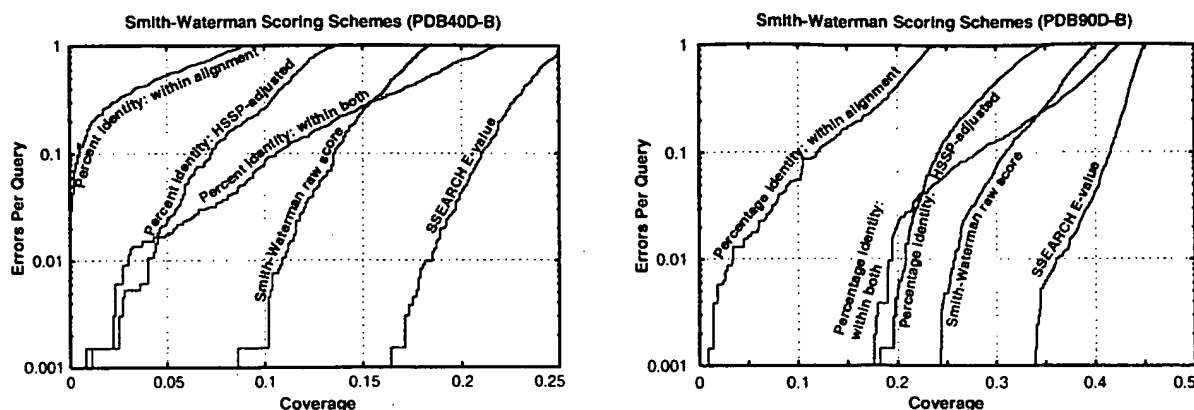


FIG. 1. Coverage vs. error plots of different scoring schemes for SSEARCH Smith-Waterman. (A) Analysis of PDB40D-B database. (B) Analysis of PDB90D-B database. All of the proteins in the database were compared with each other using the SSEARCH program. The results of this single set of comparisons were considered using five different scoring schemes and assessed. The graphs show the coverage and errors per query (EPQ) for statistical scores, raw scores, and three measures using percentage identity. In the coverage vs. error plot, the x axis indicates the fraction of all homologs in the database (known from structure) which have been detected. Precisely, it is the number of detected pairs of proteins with the same fold divided by the total number of pairs from a common superfamily. PDB40D-B contains a total of 9,044 homologs, so a score of 10% indicates identification of 904 relationships. The y axis reports the number of EPQ. Because there are 1,323 queries made in the PDB40D-B all-vs.-all comparison, 13 errors corresponds to 0.01, or 1% EPQ. The y axis is presented on a log scale to show results over the widely varying degrees of accuracy which may be desired. The scores that correspond to the levels of EPQ and coverage are shown in Fig. 4 and Table 1. The graph demonstrates the trade-off between sensitivity and selectivity. As more homologs are found (moving to the right), more errors are made (moving up). The ideal method would be in the lower right corner of the graph, which corresponds to identifying many evolutionary relationships without selecting unrelated proteins. Three measures of percentage identity are plotted. Percentage identity within alignment is the degree of identity within the aligned region of the proteins, without consideration of the alignment length. Percentage identity within both is the number of identical residues in the aligned region as a percentage of the average length of the query and target proteins. The HSP equation (17) is  $H = 290.15l^{-0.562}$  where  $l$  is length for  $10 < l < 80$ ;  $H > 100$  for  $l < 10$ ;  $H = 24.7$  for  $l > 80$ . The percentage identity HSP-adjusted score is the percent identity within the alignment minus  $H$ . Smith-Waterman raw scores and E-values were taken directly from the sequence comparison program.

perfect separation, with all of the homologs at the top of the list and unrelated proteins below. In practice, perfect separation is impossible to achieve so instead one is interested in drawing a threshold above which there are the largest number of related pairs of sequences consistent with an acceptable error rate.

Our procedure involved measuring the coverage and error for every threshold. Coverage was defined as the fraction of structurally determined homologs that have scores above the selected threshold; this reflects the sensitivity of a method. Errors per query (EPQ), an indicator of selectivity, is the number of nonhomologous pairs above the threshold divided by the number of queries. Graphs of these data, called coverage vs. error plots, were devised to understand how

protocols compare at different levels of accuracy. These graphs share effectively all of the beneficial features of Receiver Operating Characteristic (ROC) plots (33, 34) but better represent the high degrees of accuracy required in sequence comparison and the huge background of nonhomologs.

This assessment procedure is directly relevant to practical sequence database searching, for it provides precisely the information necessary to perform a reliable sequence database search. The EPQ measure places a premium on score consistency; that is, it requires scores to be comparable for different queries. Consistency is an aspect which has been largely

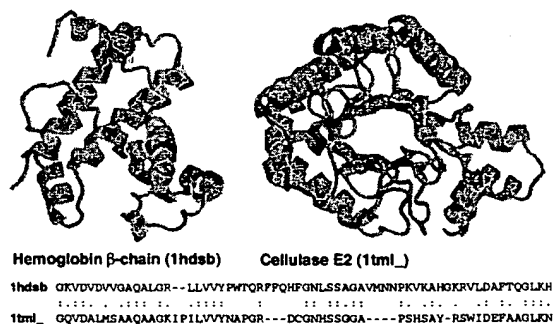


FIG. 2. Unrelated proteins with high percentage identity. Hemoglobin  $\beta$ -chain (PDB code 1hds chain b, ref. 38, *Left*) and cellulase E2 (PDB code 1tml, ref. 39, *Right*) have 39% identity over 64 residues, a level which is often believed to be indicative of homology. Despite this high degree of identity, their structures strongly suggest that these proteins are not related. Appropriately, neither the raw alignment score of 85 nor the E-value of 1.3 is significant. Proteins rendered by RASMOL (40).

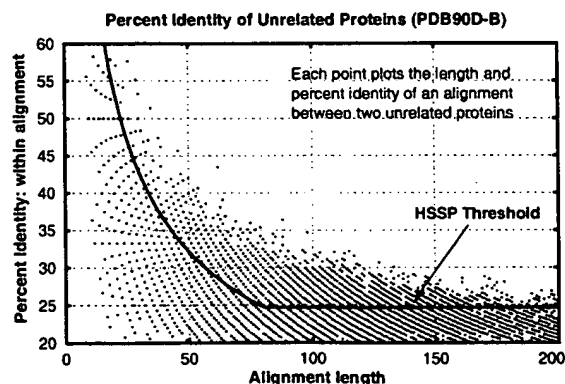


FIG. 3. Length and percentage identity of alignments of unrelated proteins in PDB90D-B: Each pair of nonhomologous proteins found with SSEARCH is plotted as a point whose position indicates the length and the percentage identity within the alignment. Because alignment length and percentage identity are quantized, many pairs of proteins may have exactly the same alignment length and percentage identity. The line shows the HSP threshold (though it is intended to be applied with a different matrix and parameters).

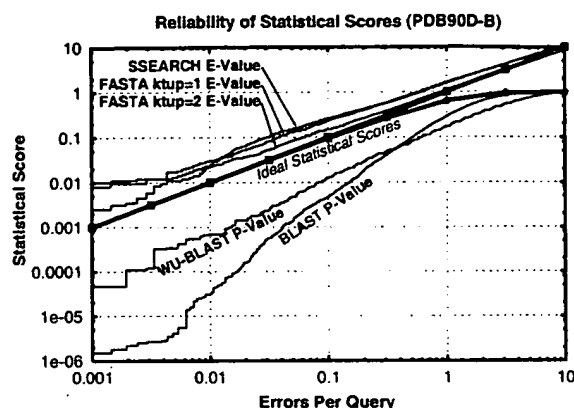


FIG. 4. Reliability of statistical scores in PDB90D-B: Each line shows the relationship between reported statistical score and actual error rate for a different program. E-values are reported for SSEARCH and FASTA, whereas P-values are shown for BLAST and WU-BLAST2. If the scoring were perfect, then the number of errors per query and the E-values would be the same, as indicated by the upper bold line. (P-values should be the same as EPQ for small numbers, and diverges at higher values, as indicated by the lower bold line.) E-values from SSEARCH and FASTA are shown to have good agreement with EPQ but underestimate the significance slightly. BLAST and WU-BLAST2 are overconfident, with the degree of exaggeration dependent upon the score. The results for PDB40D-B were similar to those for PDB90D-B despite the difference in number of homologs detected. This graph could be used to roughly calibrate the reliability of a given statistical score.

ignored in previous tests but is essential for the straightforward or automatic interpretation of sequence comparison results. Further, it provides a clear indication of the confidence that should be ascribed to each match. Indeed, the EPQ measure should approximate the expectation value reported by database searching programs, if the programs' estimates are accurate.

**The Performance of Scoring Schemes.** All of the programs tested could provide three fundamental types of scores. The first score is the percentage identity, which may be computed in several ways based on either the length of the alignment or the lengths of the sequences. The second is a "raw" or "Smith-Waterman" score, which is the measure optimized by the Smith-Waterman algorithm and is computed by summing the substitution matrix scores for each position in the alignment and subtracting gap penalties. In BLAST, a measure

related to this score is scaled into bits. Third is a statistical score based on the extreme value distribution. These results are summarized in Fig. 1.

**Sequence Identity.** Though it has been long established that percentage identity is a poor measure (35), there is a common rule-of-thumb stating that 30% identity signifies homology. Moreover, publications have indicated that 25% identity can be used as a threshold (17, 36). We find that these thresholds, originally derived years ago, are not supported by present results. As databases have grown, so have the possibilities for chance alignments with high identity; thus, the reported cutoffs lead to frequent errors. Fig. 2 shows one of the many pairs of proteins with very different structures that nonetheless have high levels of identity over considerable aligned regions. Despite the high identity, the raw and the statistical scores for such incorrect matches are typically not significant. The principal reasons percentage identity does so poorly seem to be that it ignores information about gaps and about the conservative or radical nature of residue substitutions.

From the PDB90D-B analysis in Fig. 3, we learn that 30% identity is a reliable threshold for this database only for sequence alignments of at least 150 residues. Because one unrelated pair of proteins has 43.5% identity over 62 residues, it is probably necessary for alignments to be at least 70 residues in length before 40% is a reasonable threshold, for a database of this particular size and composition.

At a given reliability, scores based on percentage identity detect just a fraction of the distant homologs found by statistical scoring. If one measures the percentage identity in the aligned regions without consideration of alignment length, then a negligible number of distant homologs are detected. Use of the HSSP equation improves the value of percentage identity, but even this measure can find only 4% of all known homologs at 1% EPQ. In short, percentage identity discards most of the information measured in a sequence comparison.

**Raw Scores.** Smith-Waterman raw scores perform better than percentage identity (Fig. 1), but ln-scaling (7) provided no notable benefit in our analysis. It is necessary to be very precise when using either raw or bit scores because a 20% change in cutoff score could yield a tenfold difference in EPQ. However, it is difficult to choose appropriate thresholds because the reliability of a bit score depends on the lengths of the proteins matched and the size of the database. Raw score thresholds also are affected by matrix and gap parameters.

**Statistical Scores.** Statistical scores were introduced partly to overcome the problems that arise from raw scores. This scoring scheme provides the best discrimination between homologous proteins and those which are unrelated. Most

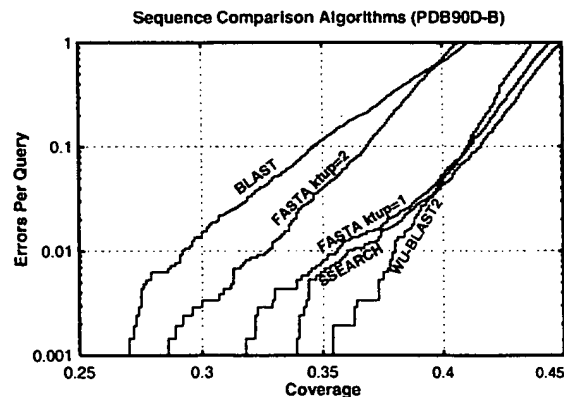
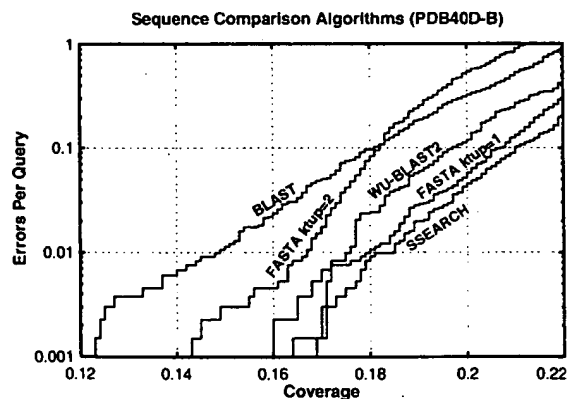


FIG. 5. Coverage vs. error plots of different sequence comparison methods: Five different sequence comparison methods are evaluated, each using statistical scores (E- or P-values). (A) PDB40D-B database. In this analysis, the best method is the slow SSEARCH, which finds 18% of relationships at 1% EPQ. FASTA ktup = 1 and WU-BLAST2 are almost as good. (B) PDB90D-B database. The quick WU-BLAST2 program provides the best coverage at 1% EPQ on this database, although at higher levels of error it becomes slightly worse than FASTA ktup = 1 and SSEARCH.

# Microarrays and Toxicology: The Advent of Toxicogenomics

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The availability of genome-scale DNA sequence information and reagents has radically altered life-science research. This revolution has led to the development of a new scientific subdiscipline derived from a combination of the fields of toxicology and genomics. This subdiscipline, termed toxicogenomics, is concerned with the identification of potential human and environmental toxicants, and their putative mechanisms of action, through the use of genomics resources. One such resource is DNA microarrays or "chips," which allow the monitoring of the expression levels of thousands of genes simultaneously. Here we propose a general method by which gene expression, as measured by cDNA microarrays, can be used as a highly sensitive and informative marker for toxicity. Our purpose is to acquaint the reader with the development and current state of microarray technology and to present our view of the usefulness of microarrays to the field of toxicology. *Mol. Carcinog.* 24:153-159, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** toxicology; gene expression; animal bioassay

## INTRODUCTION

Technological advancements combined with intensive DNA sequencing efforts have generated an enormous database of sequence information over the past decade. To date, more than 3 million sequences, totaling over 2.2 billion bases [1], are contained within the GenBank database, which includes the complete sequences of 19 different organisms [2]. The first complete sequence of a free-living organism, *Haemophilus influenzae*, was reported in 1995 [3] and was followed shortly thereafter by the first complete sequence of a eukaryote, *Saccharomyces cerevisiae* [4]. The development of dramatically improved sequencing methodologies promises that complete elucidation of the *Homo sapiens* DNA sequence is not far behind [5].

To exploit more fully the wealth of new sequence information, it was necessary to develop novel methods for the high-throughput or parallel monitoring of gene expression. Established methods such as northern blotting, RNase protection assays, S1 nuclease analysis, plaque hybridization, and slot blots do not provide sufficient throughput to effectively utilize the new genomics resources. Newer methods such as differential display [6], high-density filter hybridization [7,8], serial analysis of gene expression [9], and cDNA- and oligonucleotide-based microarray "chip" hybridization [10-12] are possible solutions to this bottleneck. It is our belief that the microarray approach, which allows the monitoring of expression levels of thousands of genes simultaneously, is a tool of unprecedented power for use in toxicology studies.

Almost without exception, gene expression is altered during toxicity, as either a direct or indirect result of toxicant exposure. The challenge facing toxicologists is to define, under a given set of experimental conditions, the characteristic and specific pattern of gene expression elicited by a given toxicant. Microarray technology offers an ideal platform for this type of analysis and could be the foundation for a fundamentally new approach to toxicology testing.

## MICROARRAY DEVELOPMENT AND APPLICATIONS

### cDNA Microarrays

In the past several years, numerous systems were developed for the construction of large-scale DNA arrays. All of these platforms are based on cDNAs or oligonucleotides immobilized to a solid support. In the cDNA approach, cDNA (or genomic) clones of interest are arrayed in a multi-well format and amplified by polymerase chain reaction. The products of this amplification, which are usually 500- to 2000-bp clones from the 3' regions of the genes of interest, are then spotted onto solid support by using high-speed robotics. By using this method, microarrays of up to 10 000 clones can be generated by spotting onto a glass substrate

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; NIEHS, National Institute of Environmental Health Sciences.

[13,14]. Sample detection for microarrays on glass involves the use of probes labeled with fluorescent or radioactive nucleotides.

Fluorescent cDNA probes are generated from control and test RNA samples in single-round reverse-transcription reactions in the presence of fluorescently tagged dUTP (e.g., Cy3-dUTP and Cy5-dUTP), which produces control and test products labeled with different fluorors. The cDNAs generated from these two populations, collectively termed the "probe," are then mixed and hybridized to the array under a glass coverslip [10,11,15]. The fluorescent signal is detected by using a custom-designed scanning confocal microscope equipped with a motorized stage and lasers for fluor excitation [10,11,15]. The data are analyzed with custom digital image analysis software that determines for each DNA feature the ratio of fluor 1 to fluor 2, corrected for local background [16,17]. The strength of this approach lies in the ability to label RNAs from control and treated samples with different fluorescent nucleotides, allowing for the simultaneous hybridization and detection of both populations on one microarray. This method eliminates the need to control for hybridization between arrays. The research groups of Drs. Patrick Brown and Ron Davis at Stanford University spearheaded the effort to develop this approach, which has been successfully applied to studies of *Arabidopsis thaliana* RNA [10], yeast genomic DNA [15], tumorigenic versus non-tumorigenic human tumor cell lines [11], human T-cells [18], yeast RNA [19], and human inflammatory disease-related genes [20]. The most dramatic result of this effort was the first published account of gene expression of an entire genome, that of the yeast *Saccharomyces cerevisiae* [21].

In an alternative approach, large numbers of cDNA clones can be spotted onto a membrane support, albeit at a lower density [7,22]. This method is useful for expression profiling and large-scale screening and mapping of genomic or cDNA clones [7,22-24]. In expression profiling on filter membranes, two different membranes are used simultaneously for control and test RNA hybridizations, or a single membrane is stripped and reprobed. The signal is detected by using radioactive nucleotides and visualized by phosphorimager analysis or autoradiography. Numerous companies now sell such cDNA membranes and software to analyze the image data [25-27].

#### Oligonucleotide Microarrays

Oligonucleotide microarrays are constructed either by spotting prefabricated oligos on a glass support [13] or by the more elegant method of direct in situ oligo synthesis on the glass surface by photolithography [28-30]. The strength of this approach lies in its ability to discriminate DNA molecules based on single base-pair difference. This allows the application of this method to the fields of medical diagnos-

tics, pharmacogenetics, and sequencing by hybridization as well as gene-expression analysis.

Fabrication of oligonucleotide chips by photolithography is theoretically simple but technically complex [29,30]. The light from a high-intensity mercury lamp is directed through a photolithographic mask onto the silica surface, resulting in deprotection of the terminal nucleotides in the illuminated regions. The entire chip is then reacted with the desired free nucleotide, resulting in selected chain elongation. This process requires only  $4n$  cycles (where  $n$  = oligonucleotide length in bases) to synthesize a vast number of unique oligos, the total number of which is limited only by the complexity of the photolithographic mask and the chip size [29,31,32].

Sample preparation involves the generation of double-stranded cDNA from cellular poly(A)<sup>+</sup> RNA followed by antisense RNA synthesis in an in vitro transcription reaction with biotinylated or fluor-tagged nucleotides. The RNA probe is then fragmented to facilitate hybridization. If the indirect visualization method is used, the chips are incubated with fluor-linked streptavidin (e.g., phycoerythrin) after hybridization [12,33]. The signal is detected with a custom confocal scanner [34]. This method has been applied successfully to the mapping of genomic library clones [35], to de novo sequencing by hybridization [28,36], and to evolutionary sequence comparison of the *BRCA1* gene [37]. In addition, mutations in the cystic fibrosis [38] and *BRCA1* [39] gene products and polymorphisms in the human immunodeficiency virus-1 clade B protease gene [40] have been detected by this method. Oligonucleotide chips are also useful for expression monitoring [33] as has been demonstrated by the simultaneous evaluation of gene-expression patterns in nearly all open reading frames of the yeast strain *S. cerevisiae* [12]. More recently, oligonucleotide chips have been used to help identify single nucleotide polymorphisms in the human [41] and yeast [42] genomes.

#### THE USE OF MICROARRAYS IN TOXICOLOGY

##### Screening for Mechanism of Action

The field of toxicology uses numerous in vivo model systems, including the rat, mouse, and rabbit, to assess potential toxicity and these bioassays are the mainstay of toxicology testing. However, in the past several decades, a plethora of in vitro techniques have been developed to measure toxicity, many of which measure toxicant-induced DNA damage. Examples of these assays include the Ames test, the Syrian hamster embryo cell transformation assay, micronucleus assays, measurements of sister chromatid exchange and unscheduled DNA synthesis, and many others. Fundamental to all of these methods is the fact that toxicity is often preceded by, and results in, alterations in gene expression. In many cases, these changes in gene expression are a

far more sensitive, characteristic, and measurable endpoint than the toxicity itself. We therefore propose that a method based on measurements of the genome-wide gene expression pattern of an organism after toxicant exposure is fundamentally informative and complements the established methods described above.

We are developing a method by which toxicants can be identified and their putative mechanisms of action determined by using toxicant-induced gene expression profiles. In this method, in one or more defined model systems, dose and time-course parameters are established for a series of toxicants within a given prototypic class (e.g., polycyclic aromatic hydrocarbons (PAHs)). Cells are then treated with these agents at a fixed toxicity level (as measured by cell survival), RNA is harvested, and toxicant-induced gene expression changes are assessed by hybridization to a cDNA microarray chip (Figure 1). We have developed a custom DNA chip, called ToxChip v1.0, specifically for this purpose and will discuss it in more detail below. The changes in gene expression induced by the test agents in the model systems are analyzed, and the common set of changes unique to that class of toxicants, termed a toxicant signature, is determined.

This signature is derived by ranking across all experiments the gene-expression data based on rela-

tive fold induction or suppression of genes in treated samples versus untreated controls and selecting the most consistently different signals across the sample set. A different signature may be established for each prototypic toxicant class. Once the signatures are determined, gene-expression profiles induced by unknown agents in these same model systems can then be compared with the established signatures. A match assigns a putative mechanism of action to the test compound. Figure 2 illustrates this signature method for different types of oxidant stressors, PAHs, and peroxisome proliferators. In this example, the unknown compound in question had a gene-expression profile similar to that of the oxidant stressors in the database. We anticipate that this general method will also reveal cross talk between different pathways induced by a single agent (e.g., reveal that a compound has both PAH-like and oxidant-like properties). In the future, it may be necessary to distinguish very subtle differences between compounds within a very large sample set (e.g., thousands of highly similar structural isomers in a combinatorial chemistry library or peptide library). To generate these highly refined signatures, standard statistical clustering techniques or principal-component analysis can be used.

For the studies outlined in Figure 2, we developed the custom cDNA microarray chip ToxChip v1.0.

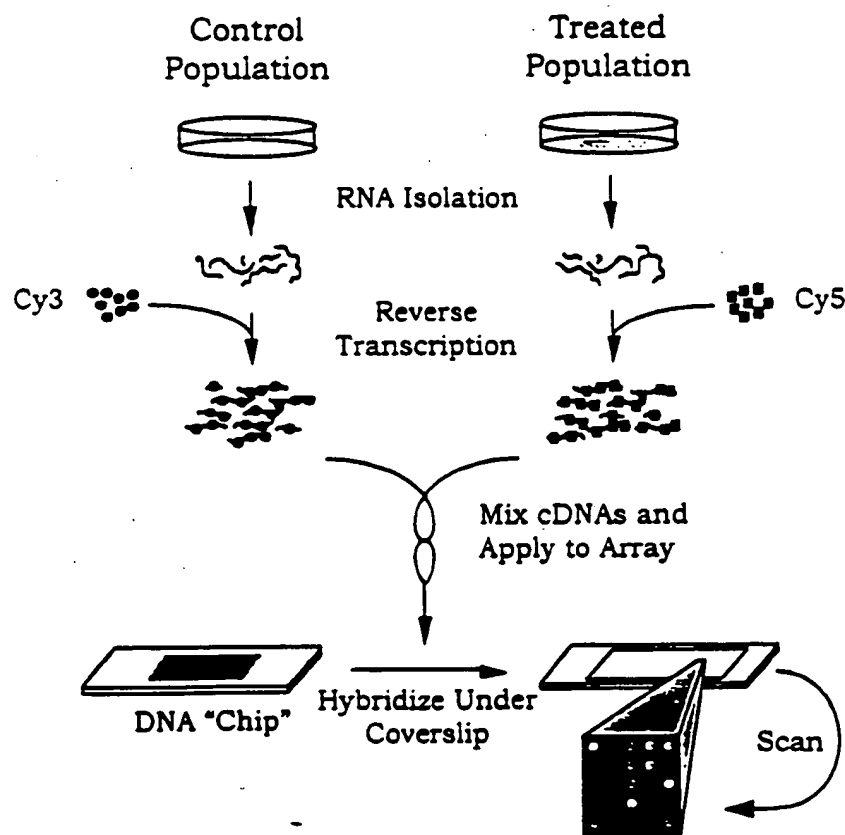


Figure 1. Simplified overview of the method for sample preparation and hybridization to cDNA microarrays. For illus-

trative purposes, samples derived from cell culture are depicted, although other sample types are amenable to this analysis.

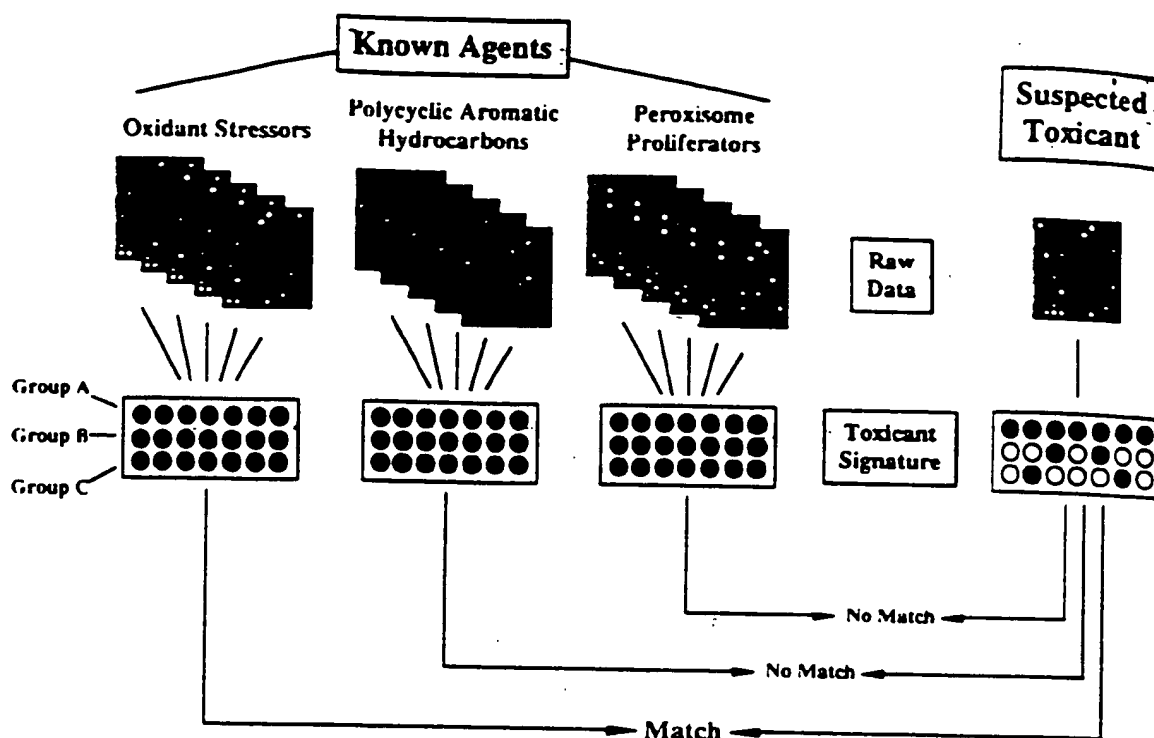


Figure 2. Schematic representation of the method for identification of a toxicant's mechanism of action. In this method, gene-expression data derived from exposure of model systems to known toxicants are analyzed, and a set of changes characteristic to that type of toxicant (termed the toxicant signature) is identified. As depicted, oxidant stressors produce

consistent changes in group A genes (indicated by red and green circles), but not group B or C genes (indicated by gray circles). The set of gene-expression changes elicited by the suspected toxicant is then compared with these characteristic patterns, and a putative mechanism of action is assigned to the unknown agent.

The 2090 human genes that comprise this subarray were selected for their well-documented involvement in basic cellular processes as well as their responses to different types of toxic insult. Included on this list are DNA replication and repair genes, apoptosis genes, and genes responsive to PAHs and dioxin-like compounds, peroxisome proliferators, estrogenic compounds, and oxidant stress. Some of the other categories of genes include transcription factors, oncogenes, tumor suppressor genes, cyclins, kinases, phosphatases, cell adhesion and motility genes, and homeobox genes. Also included in this group are 84 housekeeping genes, whose hybridization intensity is averaged and used for signal normalization of the other genes on the chip. To date, very few toxicants have been shown to have appreciable effects on the expression of these housekeeping genes. However, this housekeeping list will be revised if new data warrant the addition or deletion of a particular gene. Table 1 contains a general description of some of the different classes of genes that comprise ToxChip v1.0.

When a toxicant signature is determined, the genes within this signature are flagged within the database. When uncharacterized toxicants are then screened, the data can be quickly reformatted so that blocks of genes representing the different signatures

are displayed [11]. This facilitates rapid, visual interpretation of data. We are also developing ToxChip v2.0 and chips for other model systems, including rat, mouse, *Xenopus*, and yeast, for use in toxicology studies.

#### Animal Models in Toxicology Testing

The toxicology community relies heavily on the use of animals as model systems for toxicology testing. Unfortunately, these assays are inherently expensive, require large numbers of animals and take a long time to complete and analyze. Therefore, the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program, and the toxicology community at large are committed to reducing the number of animals used, by developing more efficient and alternative testing methodologies. Although substantial progress has been made in the development of alternative methods, bioassays are still used for testing endpoints such as neurotoxicity, immunotoxicity, reproductive and developmental toxicology, and genetic toxicology. The rodent cancer bioassay is a particularly expensive and time-consuming assay, as it requires almost 4 yr, 1200 animals, and millions of dollars to execute and analyze [43]. In vitro experiments of the type outlined in Figure 2 might provide evidence that an unknown

Table 1. ToxChip v1.0: A Human cDNA Microarray Chip Designed to Detect Responses to Toxic Insult

Gene category	No. of genes on chip
Apoptosis	72
DNA replication and repair	99
Oxidative stress/redox homeostasis	90
Peroxisome proliferator responsive	22
Dioxin/PAH responsive	12
Estrogen responsive	63
Housekeeping	84
Oncogenes and tumor suppressor genes	76
Cell-cycle control	51
Transcription factors	131
Kinases	276
Phosphatases	88
Heat-shock proteins	23
Receptors	349
Cytochrome P450s	30

\*This list is intended as a general guide. The gene categories are not unique, and some genes are listed in multiple categories.

agent is (or is not) responsible for eliciting a given biological response. This information would help to select a bioassay more specifically suited to the agent in question or perhaps suggest that a bioassay is not necessary, which would dramatically reduce cost, animal use, and time.

The addition of microarray techniques to standard bioassays may dramatically enhance the sensitivity and interpretability of the bioassay and possibly reduce its cost. Gene-expression signatures could be determined for various types of tissue-specific toxicants, and new compounds could be screened for these characteristic signatures, providing a rapid and sensitive in vivo test. Also, because gene expression is often exquisitely sensitive to low doses of a toxicant, the combination of gene-expression screening and the bioassay might allow the use of lower toxicant doses, which are more relevant to human exposure levels, and the use of fewer animals. In addition, gene-expression changes are normally measured in hours or days, not in the months to years required for tumor development. Furthermore, microarrays might be particularly useful for investigating the relationship between acute and chronic toxicity and identifying secondary effects of a given toxicant by studying the relationship between the duration of exposure to a toxicant and the gene-expression profile produced. Thus, a bioassay that incorporates gene-expression signatures with traditional endpoints might be substantially shorter, use more realistic dose regimens, and cost substantially less than the current assays do.

These considerations are also relevant for branches of toxicology not related to human health and not using rodents as model systems, such as aquatic toxicology and plant pathology. Bioassays based on the fathead minnow, *Daphnia*, and *Arabidopsis* could

also be improved by the addition of microarray analysis. The combination of microarrays with traditional bioassays might also be useful for investigating some of the more intractable problems in toxicology research, such as the effects of complex mixtures and the difficulties in cross-species extrapolation.

#### Exposure Assessment, Environmental Monitoring, and Drug Safety

The currently used methods for assessment of exposure to chemical toxicants are based on measurement of tissue toxin levels or on surrogate markers of toxicity, termed biomarkers (e.g., peripheral blood levels of hepatic enzymes or DNA adducts). Because gene expression is a sensitive endpoint, gene expression as measured with microarray technology may be useful as a new biomarker to more precisely identify hazards and to assess exposure. Similarly, microarrays could be used in an environmental-monitoring capacity to measure the effect of potential contaminants on the gene-expression profiles of resident organisms. In an analogous fashion, microarrays could be used to measure gene-expression endpoints in subjects in clinical trials. The combination of these gene-expression data and more established toxic endpoints in these trials could be used to define highly precise surrogates of safety.

Gene-expression profiles in samples from exposed individuals could be compared to the profiles of the same individuals before exposure. From this information, the nature of the toxic exposure can be determined or a relative clinical safety factor estimated. In the future it may also be possible to estimate not only the nature but the dose of the toxicant for a given exposure, based on relative gene-expression levels. This general approach may be particularly appropriate for occupational-health applications, in which unexposed and exposed samples from the same individuals may be obtainable. For example, a pilot study of gene expression in peripheral-blood lymphocytes of Polish coke-oven workers exposed to PAHs (and many other compounds) is under consideration at the NIEHS. An important consideration for these types of studies is that gene expression can be affected by numerous factors, including diet, health, and personal habits. To reduce the effects of these confounding factors, it may be necessary to compare pools of control samples with pools of treated samples. In the future it may be possible to compare exposed sample sets to a national database of human-expression data, thus eliminating the need to provide an unexposed sample from the same individual. Efforts to develop such a national gene-expression database are currently under way [44,45]. However, this national database approach will require a better understanding of genome-wide gene expression across the highly diverse human population and of the effects of environmental factors on this expression.

## Alleles, Oligo Arrays, and Toxicogenetics

Gene sequences vary between individuals, and this variability can be a causative factor in human diseases of environmental origin [46,47]. A new area of toxicology, termed toxicogenetics, was recently developed to study the relationship between genetic variability and toxicant susceptibility. This field is not the subject of this discussion, but it is worthwhile to note that the ability of oligonucleotide arrays to discriminate DNA molecules based on single base-pair differences makes these arrays uniquely useful for this type of analysis. Recent reports demonstrated the feasibility of this approach [41,42]. The NIEHS has initiated the Environmental Genome Project to identify common sequence polymorphisms in 200 genes thought to be involved in environmental diseases [48]. In a pilot study on the feasibility of this application to the Environmental Genome Project, oligonucleotide arrays will be used to resequence 20 candidate genes. This toxicogenetic approach promises to dramatically improve our understanding of interindividual variability in disease susceptibility.

## FUTURE PRIORITIES

There are many issues that must be addressed before the full potential of microarrays in toxicology research can be realized. Among these are model system selection, dose selection, and the temporal nature of gene expression. In other words, in which species, at what dose, and at what time do we look for toxicant-induced gene expression? If human samples are analyzed, how variable is global gene expression between individuals, before and after toxicant exposure? What are the effects of age, diet, and other factors on this expression? Experience, in the form of large data sets of toxicant exposures, will answer these questions.

One of the most pressing issues for array scientists is the construction of a national public database (linked to the existing public databases) to serve as a repository for gene-expression data. This relational database must be made available for public use, and researchers must be encouraged to submit their expression data so that others may view and query the information. Researchers at the National Institutes of Health have made laudable progress in developing the first generation of such a database [44,45]. In addition, improved statistical methods for gene clustering and pattern recognition are needed to analyze the data in such a public database.

The proliferation of different platforms and methods for microarray hybridizations will improve sample handling and data collection and analysis and reduce costs. However, the variety of microarray methods available will create problems of data compatibility between platforms. In addition, the near-infinite variety of experimental conditions under

which data will be collected by different laboratories will make large-scale data analysis extremely difficult. To help circumvent these future problems, a set of standards to be included on all platforms should be established. These standards would facilitate data entry into the national database and serve as reference points for cross-platform and interlaboratory data analysis.

Many issues remain to be resolved, but it is clear that new molecular techniques such as microarray hybridization will have a dramatic impact on toxicology research. In the future, the information gathered from microarray-based hybridization experiments will form the basis for an improved method to assess the impact of chemicals on human and environmental health.

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## Application of DNA Arrays to Toxicology

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DNA array technology makes it possible to rapidly genotype individuals or quantify the expression of thousands of genes on a single filter or glass slide, and holds enormous potential in toxicologic applications. This potential led to a U.S. Environmental Protection Agency-sponsored workshop titled "Application of Microarrays to Toxicology" on 7-8 January 1999 in Research Triangle Park, North Carolina. In addition to providing state-of-the-art information on the application of DNA or gene microarrays, the workshop catalyzed the formation of several collaborations, committees, and user's groups throughout the Research Triangle Park area and beyond. Potential application of microarrays to toxicologic research and risk assessment include genome-wide expression analyses to identify gene-expression networks and toxicant-specific signatures that can be used to define mode of action, for exposure assessment, and for environmental monitoring. Arrays may also prove useful for monitoring genetic variability and its relationship to toxicant susceptibility in human populations. **Key words:** DNA arrays, gene arrays, microarrays, toxicology. *Environ Health Perspect* 107:681-685 (1999). [Online 6 July 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p681-685rockett/abstract.html>

Decoding the genetic blueprint is a dream that offers manifold returns in terms of understanding how organisms develop and function in an often hostile environment. With the rapid advances in molecular biology over the last 30 years, the dream has come a step closer to reality. Molecular biologists now have the ability to elucidate the composition of any genome. Indeed, almost 20 genomes have already been sequenced and more than 60 are currently under way. Foremost among these is the Human Genome Mapping Project. However, the genomes of a number of commonly used laboratory species are also under intensive investigation, including yeast, *Arabidopsis*, maize, rice, zebra fish, mouse, rat, and dog. It is widely expected that the completion of such programs will facilitate the development of many powerful new techniques and approaches to diagnosing and treating genetically and environmentally induced diseases which afflict mankind. However, the vast amount of data being generated by genome mapping will require new high-throughput technologies to investigate the function of the millions of new genes that are being reported. Among the most widely heralded of the new functional genomics technologies are DNA arrays, which represent perhaps the most anticipated new molecular biology technique since polymerase chain reaction (PCR).

Arrays enable the study of literally thousands of genes in a single experiment. The potential importance of arrays is enormous and has been highlighted by the recent publication of an entire *Nature Genetics* supplement dedicated to the technology (1). Despite this huge surge of interest, DNA arrays are still little used and largely unproven, as demonstrated by the high ratio of review and press articles to actual data papers. Even so, the potential they offer

has driven venture capitalists into a frenzy of investment and many new companies are springing up to claim a share of this rapidly developing market.

The U.S. Environmental Protection Agency (EPA) is interested in applying DNA array technology to ongoing toxicologic studies. To learn more about the current state of the technology, the Reproductive Toxicology Division (RTD) of the National Health and Environmental Effects Research Laboratory (NHEERL; Research Triangle Park, NC) hosted a workshop on "Application of Microarrays to Toxicology" on 7-8 January 1999 in Research Triangle Park, North Carolina. The workshop was organized by David Dix, Robert Kavlock, and John Rockett of the RTD/NHEERL. Twenty-two intramural and extramural scientists from government, academia, and industry shared information, data, and opinions on the current and future applications for this exciting new technology. The workshop had more than 150 attendees, including researchers, students, and administrators from the EPA, the National Institute of Environmental Health Sciences (NIEHS), and a number of other establishments from Research Triangle Park and beyond. Presentations ranged from the technology behind array production through the sharing of actual experimental data and projections on the future importance and applications of arrays. The information contained in the workshop presentations should provide aid and insight into arrays in general and their application to toxicology in particular.

### Array Elements

In the context of molecular biology, the word "array" is normally used to refer to a series of DNA or protein elements firmly attached in

a regular pattern to some kind of supportive medium. DNA array is often used interchangeably with gene array or microarray. Although not formally defined, microarray is generally used to describe the higher density arrays typically printed on glass chips. The DNA elements that make up DNA arrays can be oligonucleotides, partial gene sequences, or full-length cDNAs. Companies offering pre-made arrays that contain less than full-length clones normally use regions of the genes which are specific to that gene to prevent false positives arising through cross-hybridization. Sequence verification of cDNA clone identity is necessary because of errors in identifying specific clones from cDNA libraries and databases. Premade DNA arrays printed on membranes are currently or imminently available for human, mouse, and rat. In most cases they contain DNA sequences representing several thousand different sequence clusters or genes as delineated through the National Center for Biotechnology Information UniGene Project (2). Many of these different UniGene clusters (putative genes) are represented only by expressed sequence tags (ESTs).

### Array Printing

Arrays are typically printed on one of two types of support matrix. Nylon membranes are used by most off-the-shelf array providers such as Clontech Laboratories, Inc. (Palo Alto, CA), Genome Systems, Inc. (St. Louis, MO), and Research Genetics, Inc. (Huntsville, AL). Microarrays such as those produced by Affymetrix, Inc. (Santa Clara, CA), Incyte Pharmaceuticals, Inc. (Palo Alto, CA), and many do-it-yourself (DIY) arraying groups use glass wafers or slides. Although standard microscope slides may be used, they must be preprepared to facilitate sticking of the DNA to the glass. Several different

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coatings have been successfully used, including silane and lysine. The coating of slides can easily be carried out in the laboratory, but many prefer the convenience of precoated slides available from suppliers.

Once the support matrix has been prepared, the DNA elements can be applied by several methods. Affymetrix, Inc., has developed a unique photolithographic technology for attaching oligonucleotides to glass wafers. More commonly, DNA is applied by either noncontact or contact printing. Noncontact printers can use thermal, solenoid, or piezoelectric technology to spray aliquots of solution onto the support matrix and may be used to produce slide or membrane-based arrays. Cartesian Technologies, Inc. (Irvine, CA) has developed nQUAD technology for use in its PixSys printers. The system couples a syringe pump with the microsolonoid valve, a combination that provides rapid quantitative dispensing of nanoliter volumes (down to 4.2 nL) over a variable volume range. A different approach to noncontact printing uses a solid pin and ring combination (Genetic Microsystems, Inc., Woburn, MA). This system (Figure 1) allows a broader range of sample, including cell suspensions and particulates, because the printing head cannot be blocked up in the same way as a spray nozzle. Fluid transfer is controlled in this system primarily by the pin dimensions and the force of deposition, although the nature of the support matrix and the sample will also affect transfer to some degree.

In contact printing, the pin head is dipped in the sample and then touched to the support matrix to deposit a small aliquot. Split pins were one of the first contact-printing devices to be reported and are the suggested format for DIY arrayers, as described by Brown (3). Split pins are small metal pins with a precise groove cut vertically in the middle of the pin tip. In this system, 1–48 split pins are positioned in the pin-head. The split pins work by simple capillary action, not unlike a fountain pen—when the pin heads are dipped in the sample, liquid is drawn into the pin groove. A small (fixed) volume is then deposited each time the split pins are gently touched to the support matrix. Sample (100–500 pL depending on a variety of parameters) can be deposited on multiple slides before refilling is required, and array densities of  $> 2,500$  spots/cm<sup>2</sup> may be produced. The deposit volume depends on the split size, sample fluidity, and the speed of printing. Split pins are relatively simple to produce and can be made in-house if a suitable machine shop is available. Alternatively, they can be obtained directly from companies such as TeleChem International, Inc. (Sunnyvale, CA).

Irrespective of their source, printers should be run through a preprint sequence prior to producing the actual experimental

arrays: the first 100 or so spots of a new run tend to be somewhat variable. Factors affecting spot reproducibility include slide treatment homogeneity, sample differences, and instrument errors. Other factors that come into play include clean ejection of the drop and clogging (nQUAD printing) and mechanical variations and long-term alteration in print-head surface of solid and split pins. However, with careful preparation it is possible to get a coefficient of variance for spot reproducibility below 10%.

One potential printing problem is sample carryover. Repeated washing, blotting, and drying (vacuum) of print pins between samples is normally effective at reducing sample carryover to negligible amounts. Printing should also be carried out in a controlled environment. Humidified chambers are available in which to place printers. These help prevent dust contamination and produce a uniform drying rate, which is important in determining spot size, quality, and reproducibility.

In summary, although several printing technologies are available, none are particularly outstanding and the bottom line is that they are still in a relatively early stage of evolution.

### Array Hybridization

The hybridization protocol is, practically speaking, relatively straightforward and those with previous experience in blotting should have little difficulty. Array hybridizations are, in essence, reverse Southern/Northern blots—instead of applying a labeled probe to the target population of DNA/RNA, the labeled population is applied to the probe(s). With membrane-based arrays, the control and treated mRNA populations are normally converted to cDNA and labeled with isotope (e.g., <sup>32</sup>P) in the process. These labeled populations are then hybridized independently to parallel or serial arrays and the hybridization signal is detected with a phosphorimager. A less commonly used alternative to radioactive probes is enzymatic detection. The probe may be biotinylated, haptenylated, or have alkaline phosphatase/horseradish peroxidase attached. Hybridization is detected by enzymatic reaction yielding a color reaction (4). Differences in hybridization signals can be detected by eye or, more accurately, with the help of digital imaging and commercially available software. The labeling of the test populations for slide-based microarrays uses a slightly different approach. The probe typically consists of two samples of poly(A)<sup>+</sup> RNA (usually from a treated and a control population) that are converted to cDNA; in the process each is labeled with a different fluor. The independently labeled probes are then mixed together and hybridized to a single microarray slide and the resulting combined fluorescent signal is scanned. After

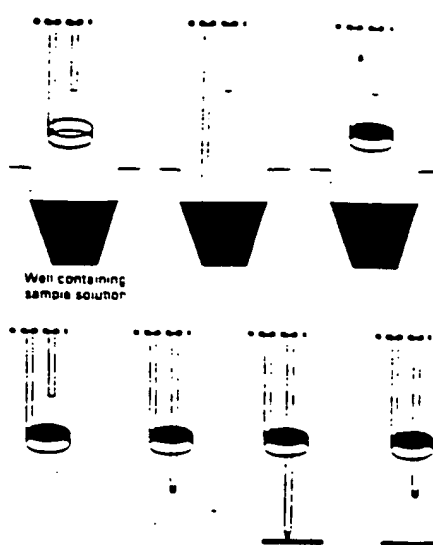


Figure 1. Genetic Microsystems (Woburn, MA) pin ring system for printing arrays. The pin ring combination consists of a circular open ring oriented parallel to the sample solution, with a vertical pin centered over the ring. When the ring is dipped into a solution and lifted, it withdraws an aliquot of sample held by surface tension. To spot the sample, the pin is driven down through the ring and a portion of the solution is transferred to the bottom of the pin. The pin continues to move downward until the pendant drop of solution makes contact with the underlying surface. The pin is then lifted, and gravity and surface tension cause deposition of the spot onto the array. Figure from Flowers et al. (14), with permission from Genetic Microsystems.

normalization, it is possible to determine the ratio of fluorescent signals from a single hybridization of a slide-based microarray.

cDNA derived from control and treated populations of RNA is most commonly hybridized to arrays, although subtractive hybridization or differential display reactions may also be used. Fluorophore- or radiolabeled nucleotides are directly incorporated into the cDNA in the process of converting RNA to cDNA. Alternatively, 5' end-labeled primers may be used for cDNA synthesis. These are labeled with a fluorophore for direct visualization of the hybridized array. Alternatively, biotin or a hapten may be attached to the primer, in which case fluor-labeled streptavidin or antibody must be applied before a signal can be generated. The most commonly used fluorophores at present are cyanine (Cy)3 and Cy5 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). However, the relative expense of these fluorescent conjugates has driven a search for cheaper alternatives. Fluorescein, rhodamine, and Texas red have all been used, and companies such as Molecular Probes, Inc. (Eugene, OR) are developing a series of labeled nucleotides with a wide range of excitation and emission spectra which may prove to function as well as the Cy dyes.

## Analysis of DNA Microarrays

Membrane-based arrays are normally analyzed on film or with a phosphorimager, whereas chip-based arrays require more specialized scanning devices. These can be divided into three main groups: the charge-coupled device camera systems, the nonconfocal laser scanners, and the confocal laser scanners. The advantages and disadvantages of each system are listed in Table 1.

Because a typical spot on a microarray can contain  $> 10^6$  molecules, it is clear that a large variation in signal strength may occur. Current scanners cannot work across this many orders of magnitude (4 or 5 is more typical). However, the scanning parameters can normally be adjusted to collect more or less signal, such that two or three scans of the same array should permit the detection of rare and abundant genes.

When a microarray is scanned, the fluorescent images are captured by software normally included with the scanner. Several commercial suppliers provide additional software for quantifying array images, but the software tools are constantly evolving to meet the developing needs of researchers, and it is prudent to define one's own needs and clarify the exact capabilities of the software before its purchase. Issues that should be considered include the following:

- Can the software locate offset spots?
- Can it quantitate across irregular hybridization signals?
- Can the arrayed genes be programmed in for easy identification and location?
- Can the software connect via the Internet to databases containing further information on the gene(s) of interest?

One of the key issues raised at the workshop was the sensitivity of microarray technology. Experiments by General Scanning, Inc. (Watertown, MA), have shown that by using the Cy dyes and their scanner, signal can be detected down to levels of  $< 1$  fluor molecule per square micrometer, which translates to detecting a rare message at approximately one copy per cell or less.

## Array Applications

Although arrays are an emerging technology certain to undergo improvement and alteration, they have already been applied usefully to a number of model systems. Arrays are at their most powerful when they contain the entire genome of the species they are being used to study. For this reason, they have strong support among researchers utilizing yeast and *Caenorhabditis elegans* (5). The genomes of both of these species have been sequenced and, in the case of yeast, deposited onto arrays for examination of gene expression (6,7). With both of these species, it is relatively easy to perturb individual gene expression. Indeed, C

Table 1. Advantages and disadvantages of different microarray scanning systems

	CCD camera system	Nonconfocal laser scanner	Confocal laser scanner
Advantages	Few moving parts  Fast scanning of bright samples	Relatively simple optics —	Small depth of focus reduces artifacts May have high light collection efficiency
Disadvantages	Less appropriate for dim samples Optical scanner can limit performance	Low light collection efficiency Background artifacts not rejected  Resolution typically low	Small depth of focus requires scanning precision

CCD, charge-coupled device.  
From Kawasaki (13).

*elegans* knockouts can be made simply by soaking the worms in an antisense solution of the gene to be knocked out.

By a process of systematic gene disruption, it is now possible to examine the cause and effect relationships between different genes in these simple organisms. This kind of approach should help elucidate biochemical pathways and genetic control processes, deconvolute polygenic interactions, and define the architecture of the cellular network. A simple case study of how this can be achieved was presented by Butow [University of Texas Southwestern Medical Center, Dallas, TX (Figure 2)]. Although it is the phenotypic result of a single gene knockout that is being examined, the effect of such perturbation will almost always be polygenic. Polygenic interactions will become increasingly important as researchers begin to move away from single gene systems when examining the nature of toxicologic responses to external stimuli. This is especially important in toxicology because the phenotype produced by a given environmental insult is never the result of the action of a single gene; rather, it is a complex interaction of one or multiple cellular pathways. Phenomena such as quantitative trait (the continuous variation of phenotype), epistasis (the effect of alleles of one or more genes on the expression of other genes), and penetrance (proportion of individuals of a given genotype that display a particular phenotype) will become increasingly evident and important as toxicologists push toward the ultimate goal of matching the responses of individuals to different environmental stimuli.

Analysis of the transcriptome (the expression level of all the genes in a given cell population) was a use of arrays addressed by several speakers. Unfortunately, current gene nomenclature is often confusing in that single genes are allocated multiple names (usually as a result of independent discovery by different laboratories), and there was a call for standardization of gene nomenclature. Nevertheless, once a transcriptome has been assembled it can then be transferred onto arrays and used to screen any chosen system. The EPA MicroArray Consortium (EPAMAC) is assembling testes

transcriptomes for human, rat, and mouse. In a slightly different approach, Nuwaysir et al. (8) describes how the NIEHS assembled what is effectively a "toxicological transcriptome"—a library of human and mouse genes that have previously been proven or implicated in responses to toxicologic insults. Clontech Laboratories, Inc. (Palo Alto, CA), has begun a similar process by developing stress/toxicology filter arrays of rat, mouse, and human genes. Thus, rather than being tissue or cell specific, these stress/toxicology arrays can be used across a variety of model systems to look for alterations in the expression of toxicologically important genes and define the new field of toxicogenomics. The potential to identify toxicant families based on tissue- or cell-specific gene expression could revolutionize drug testing. These molecular signatures or fingerprints could not only point to the possible toxicity/carcinogenicity of newly discovered compounds (Figure 3), but also aid in elucidating their mechanism of action through identification of gene expression networks. By extension, such signatures could provide easily identifiable biomarkers to assess the degree, time, and nature of exposure.

DNA arrays are primarily a tool for examining differential gene expression in a given model. In this context they are referred to as closed systems because they lack the ability of other differential expression technologies, e.g., differential display and subtractive hybridization, to detect previously unknown genes not present on the array. This would appear to limit the power of DNA arrays to the imaginations and preconceptions of the researcher in selecting genes previously characterized and thought to be involved in the model system. However, the various genome sequencing projects have created a new category of sequence—the EST—that has partially mollified this deficiency. ESTs are cDNAs expressed in a given tissue that, although they may share some degree of sequence similarity to previously characterized genes, have not been assigned specific genetic identity. By incorporating EST clones into an array, it is possible to monitor the expression of these unknown genes. This can enable the identification of previously uncharacterized genes that may have biologic

significance in the model system. Filter arrays from Research Genetics and slide arrays from InCyte Pharmaceuticals both incorporate large numbers of ESTs from a variety of species.

A further use of microarrays is the identification of single nucleotide polymorphisms (SNPs). These genomic variations are abundant—they occur approximately every 1 kb or so—and are the basis of restriction fragment length polymorphism analysis used in forensic analysis. Affymetrix, Inc., designed chips that contain multiple repeats of the same gene sequence. Each position is present with all four possible bases. After the hybridization of the sample, the degree of hybridization to the different sequences can be measured and the exact sequence of the target gene deduced. SNPs are thought to be of vital importance in drug metabolism and toxicology. For example, single base differences in the regulatory region or active site of some genes can account for huge differences in the activity of that gene. Such SNPs are thought to explain why some people are able to metabolize certain xenobiotics better than others. Thus, arrays provide a further tool for the toxicologist investigating the nature of susceptible subpopulations and toxicologic response.

There are still many wrinkles to be ironed out before arrays become a standard tool for toxicologists. The main issues raised at the workshop by those with hands-on experience were the following:

- Expense: the cost of purchasing/contracting this technology is still too great for many individual laboratories.

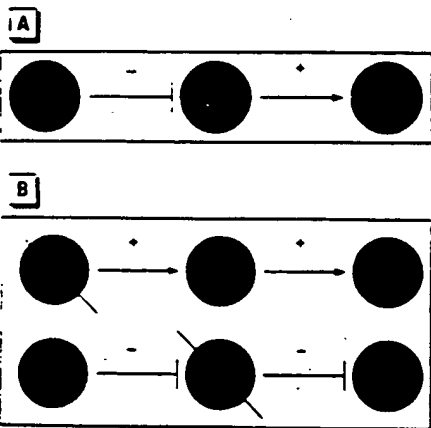


Figure 2. Potential effects of gene knockout within positively and negatively regulated gene expression networks.  $i_1$  is limiting in wild type for expression of  $i_2$ . (A) A simple, two-component, linear regulatory network operating on gene  $i_2$ , where  $i_1$  is a positive effector of  $i_2$  and  $j_1$  is either a positive or negative effector of  $i_1$ . This network could be deduced by examining the consequence of (B) deleting  $j_1$  on the expression of  $i_1$  and  $i_2$ , where the expression of  $i_2$  would be decreased or increased depending on whether  $j_1$  was a positive or negative regulator. These and other connected components of even greater complexity could be revealed by genome-wide expression analysis. From Butow (15).

- Clones: the logistics of identifying, obtaining, and maintaining a set of nonredundant, non-contaminated, sequence-verified, species/cell/tissue/field-specific clones.
- Use of inbred strains: where whole-organism models are being used, the use of inbred strains is important to reduce the potentially confusing effects of the individual variation typically seen in outbred populations.
- Probe: the need for relatively large amounts of RNA, which limits the type of sample (e.g., biopsy) that can be used. Also, different RNA extraction methods can give different results.
- Specificity: the ability to discriminate accurately between closely related genes (e.g., the cytochrome p450 family) and splice variants.
- Quantitation: the quantitation of gene expression using gene arrays is still open to debate. One reason for this is the different incorporation of the labeling dyes. However, the main difficulty lies in knowing what to normalize against. One option is to include a large number of so-called housekeeping genes in the array. However, the expression of these genes often change depending on the tissue and the toxicant, so it is necessary to characterize the expression of these genes in the model system before utilizing them. This is clearly not a viable option when screening multiple new compounds. A second option is to include on the array genes from a nonrelated species (e.g., a plant gene on an animal array) and to spike the probe with synthetic RNA(s) complementary to the gene(s).
- Reproducibility: this is sometimes questionable, and a figure of approximately two or three repeats was used as the minimum number required to confirm initial findings.

Again, however, most people advocated the use of Northern blots or reverse transcriptase PCR to confirm findings.

- Sensitivity: concerns were voiced about the number of target molecules that must be present in a sample for them to be detected on the array.
- Efficiency: reproducible identification of 1.5- to 2-fold differences in expression was reported, although the number of genes that undergo this level of change and remain undetected is open to debate. It is important that this level of detection be ultimately achieved because it is commonly perceived that some important transcription factors and their regulators respond at such low levels. In most cases, 3- to 5-fold was the minimum change that most were happy to accept.
- Bioinformatics: perhaps the greatest concern was how to accurately interpret the data with the greatest accuracy and efficiency. The biggest headache is trying to identify networks of gene expression that are common to different treatments or doses. The amount of data from a single experiment is huge. It may be that, in the future, several groups individually equipped with specialized software algorithms for studying their favorite genes or gene systems will be able to share the same hybridized chips. Thus, arrays could usher in a new perspective on collaboration and the sharing of data.

## EPAMAC

Perhaps the main reason most scientists are unable to use array technology is the high cost involved, whether buying off-the-shelf membranes, using contract printing services, or

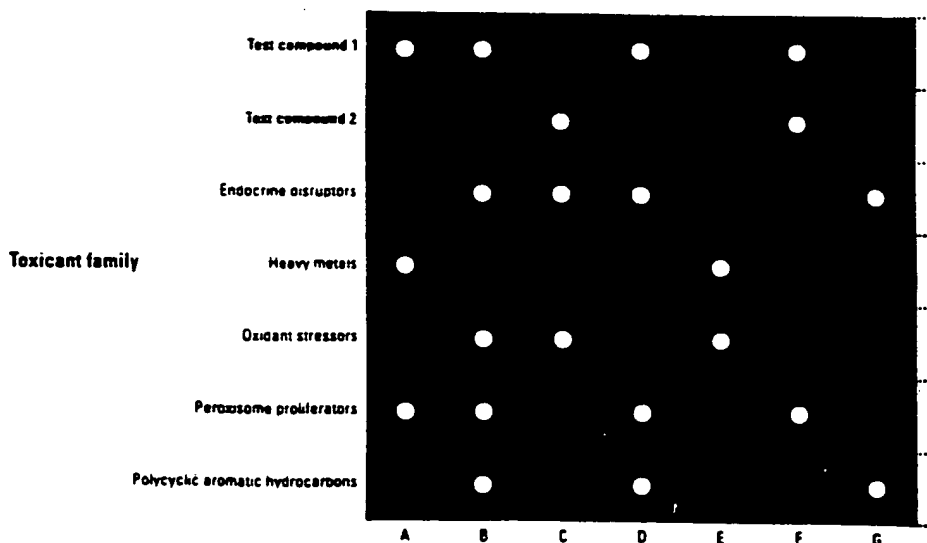


Figure 3. Gene expression profiles—also called fingerprints or signatures—of known toxicants or toxicant families may, in the future, be used to identify the potential toxicity of new drugs, etc. In this example, the genetic signature of test compound 1 is identical to that of known peroxisome proliferators, whereas that of test compound 2 does not match any known toxicant family. Based on these results, test compound 2 would be retained for further testing and test compound 1 would be eliminated.

producing chips in-house. In view of this, researchers at the RTD/NHEERL initiated the EPAMAC. This consortium brings together scientists from the EPA and a number of extramural labs with the aim of developing microarray capability through the sharing of resources and data. EPAMAC researchers are primarily interested in the developmental and toxicologic changes seen in testicular and breast tissue, and a portion of the workshop was set aside for EPAMAC members to share their ideas on how the experimental application of microarrays could facilitate their research. One of the central areas of interest to EPAMAC members is the effect of xenobiotics on male fertility and reproductive health. Of greatest concern is the effect of exposure during critical periods of development and germ cell differentiation (9), and how this may compromise sperm counts and quality following sexual maturation (10). As well as spermatogenic tissue, there is also interest in how residual mRNA found in mature sperm (11) could be used as an indicator of previous xenobiotic effects (it is easier to obtain a semen sample than a testicular biopsy). Arrays will be used to examine and compare the effect of exposure to heat and chemicals in testicular and epididymal gene expression profiles, with the aim of establishing relationships/associations between changes in developmental landmarks and the effects on sperm count and quality. Cluster, pattern, and other analysis of such data should help identify hidden relationships between genes that may reveal potential mechanisms of action and uncover roles for genes with unknown functions.

## Summary

The full impact of DNA arrays may not be seen for several years, but the interest shown at this regional workshop indicates the high level of interest that they foster. Apart from educating and advertising the various technologies in this field, this workshop brought together a number of researchers from the Research Triangle Park area who are already using DNA arrays. The interest in sharing ideas and experiences led to the initiation of a Triangle array user's group.

Array technology is still in its infancy. This means that the hardware is still improving and there is no current consensus for standard procedures, quantitation, and interpretation. Consistency in spotting and scanning arrays is not yet optimized, and this is one of the most critical requirements of any experiment. In addition, one of the dark regions of array technology—strife in the courts over who owns what portions of it—has further muddled the future and is a potential barrier toward the development of consensus procedures.

Perhaps the greatest hurdle for the application of arrays is the actual interpretation of data. No specialists in bioinformatics attended the workshop, largely because they are rare and because as yet no one seems clear on the best method of approaching data analysis and interpretation. Cross-referencing results from multiple experiments (time, dose, repeats, different animals, different species) to identify commonly expressed genes is a great challenge. In most cases, we are still a long way from understanding how the expression of gene *X* is related to the expression of gene *Y*, and ordering gene expression to delineate causal relationships.

To the ordinary scientist in the typical laboratory, however, the most immediate problem is a lack of affordable instrumentation. One can purchase premade membranes at relatively affordable prices. Although these may be useful in identifying individual genes to pursue in more detail using other methods, the numbers that would be required for even a small routine toxicology experiment prohibit this as a truly viable approach. For the toxicologist, there is a need to carry out multiple experiments—dose responses, time curves, multiple animals, and repeats. Glass-based DNA arrays are most attractive in this context because they can be prepared in large batches from the same DNA source and accommodate control and treated samples on the same chip. Another problem with current off-the-shelf arrays is that they often do not contain one or more of the particular genes a group is interested in. One alternative is to obtain and/or produce a set of custom clones and have contract printing of membranes or slides carried out by a company such as Genomic Solutions, Inc. (Ann Arbor, MI). This approach

is less expensive than having our own, or one's own entire system, although at some point it might make economic sense to print one's own arrays.

Finally, DNA arrays are currently a team effort. They are a technology that uses a wide range of skills including engineering, statistics, molecular biology, chemistry, and bioinformatics. Because most individuals are skilled in only one or perhaps two of these areas, it appears that success with arrays may be best expected by teams of collaborators consisting of individuals having each of these skills.

Those considering array applications may be amused or goaded on by the following quote from *Fortune* magazine (12):

Microprocessors have reshaped our economy, spawned vast fortunes and changed the way we live. Gene chips could be even bigger.

Although this comment may have been designed to excite the imagination rather than accurately reflect the truth, it is fair to say that the age of functional genomics is upon us. DNA arrays look set to be an important tool in this new age of biotechnology and will likely contribute answers to some of toxicology's most fundamental questions.

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## Expression profiling in toxicology — potentials and limitations

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### Abstract

Recent progress in genomics and proteomics technologies has created a unique opportunity to significantly impact the pharmaceutical drug development processes. The perception that cells and whole organisms express specific inducible responses to stimuli such as drug treatment implies that unique expression patterns, molecular fingerprints, indicative of a drug's efficacy and potential toxicity are accessible. The integration into state-of-the-art toxicology of assays allowing one to profile treatment-related changes in gene expression patterns promises new insights into mechanisms of drug action and toxicity. The benefits will be improved lead selection, and optimized monitoring of drug efficacy and safety in pre-clinical and clinical studies based on biologically relevant tissue and surrogate markers. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Proteomics; Genomics; Toxicology

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### 1. Introduction

The majority of drugs act by binding to protein targets, most to known proteins representing enzymes, receptors and channels, resulting in effects such as enzyme inhibition and impairment of signal transduction. The treatment-induced perturbations provoke feedback reactions aiming to compensate for the stimulus, which almost always are associated with signals to the nucleus, resulting in altered gene expression. Such gene expression regulations account for both the

pharmacological action and the toxicity of a drug and can be visualized by either global mRNA or global protein expression profiling. Hence, for each individual drug, a characteristic gene regulation pattern, its molecular fingerprint, exists which bears valuable information on its mode of action and its mechanism of toxicity.

Gene expression is a multistep process that results in an active protein (Fig. 1). There exist numerous regulation systems that exert control at and after the transcription and the translation step. Genomics, by definition, encompasses the quantitative analysis of transcripts at the mRNA level, while the aim of proteomics is to quantify gene expression further down-stream, creating a snapshot of gene regulation closer to ultimate cell function control.

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## 2. Global mRNA profiling

Expression data at the mRNA level can be produced using a set of different technologies such as DNA microarrays, reverse transcript imaging, amplified fragment length polymorphism (AFLP), serial analysis of gene expression (SAGE) and others. Currently, DNA microarrays are very popular and promise a great potential. On a typical array, each gene of interest is represented either by a long DNA fragment (200–2400 bp) typically generated by polymerase chain reaction (PCR) and spotted on a suitable substrate using robotics (Schena et al., 1995; Shalon et al., 1996) or by several short oligonucleotides (20–30 bp) synthesized directly onto a solid support using photolabile nucleotide chemistry (Fodor et al., 1991; Chee et al., 1996). From control and treated tissues, total RNA or mRNA is isolated and reverse transcribed in the presence of radioactive or fluorescent labeled nucleotides, and the labeled probes are then hybridized to the arrays. The intensity of the array signal is measured for each gene transcript by either autoradiography or laser scanning confocal microscopy. The ratio between the signals of control and treated samples reflect the relative drug-induced change in transcript abundance.

## 3. Global protein profiling

Global quantitative expression analysis at the protein level is currently restricted to the use of two-dimensional gel electrophoresis. This technique combines separation of tissue proteins by isoelectric focusing in the first dimension and by sodium dodecyl sulfate slab gel electrophoresis-based molecular weight separation on the second, orthogonal dimension (Anderson et al., 1991). The product is a rectangular pattern of protein spots that are typically revealed by Coomassie Blue, silver or fluorescent staining (Fig. 2). Protein spots are identified by mass spectrometry following generation of peptide mass fingerprints (Mann et al., 1993) and sequence tags (Wilkins et al., 1996). Similar to the mRNA approach, the ratio between the optical density of spots from control and treated samples are compared to search for treatment-related changes.

## 4. Expression data analysis

Bioinformatics forms a key element required to organize, analyze and store expression data from either source, the mRNA or the protein level. The overall objective, once a mass of high-quality

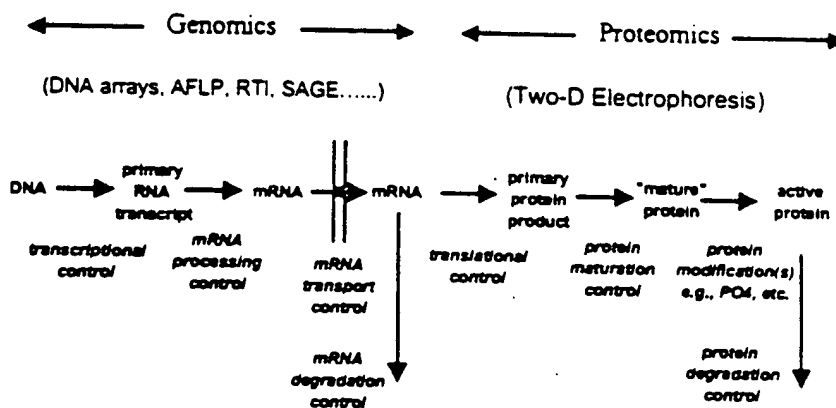


Fig. 1. Production of an active protein is a multistep process in which numerous regulation systems exert control at various stages of expression. Molecular fingerprints of drugs can be visualized through expression profiling at the mRNA level (genomics) using a variety of technologies and at the protein level (proteomics) using two-dimensional gel electrophoresis.

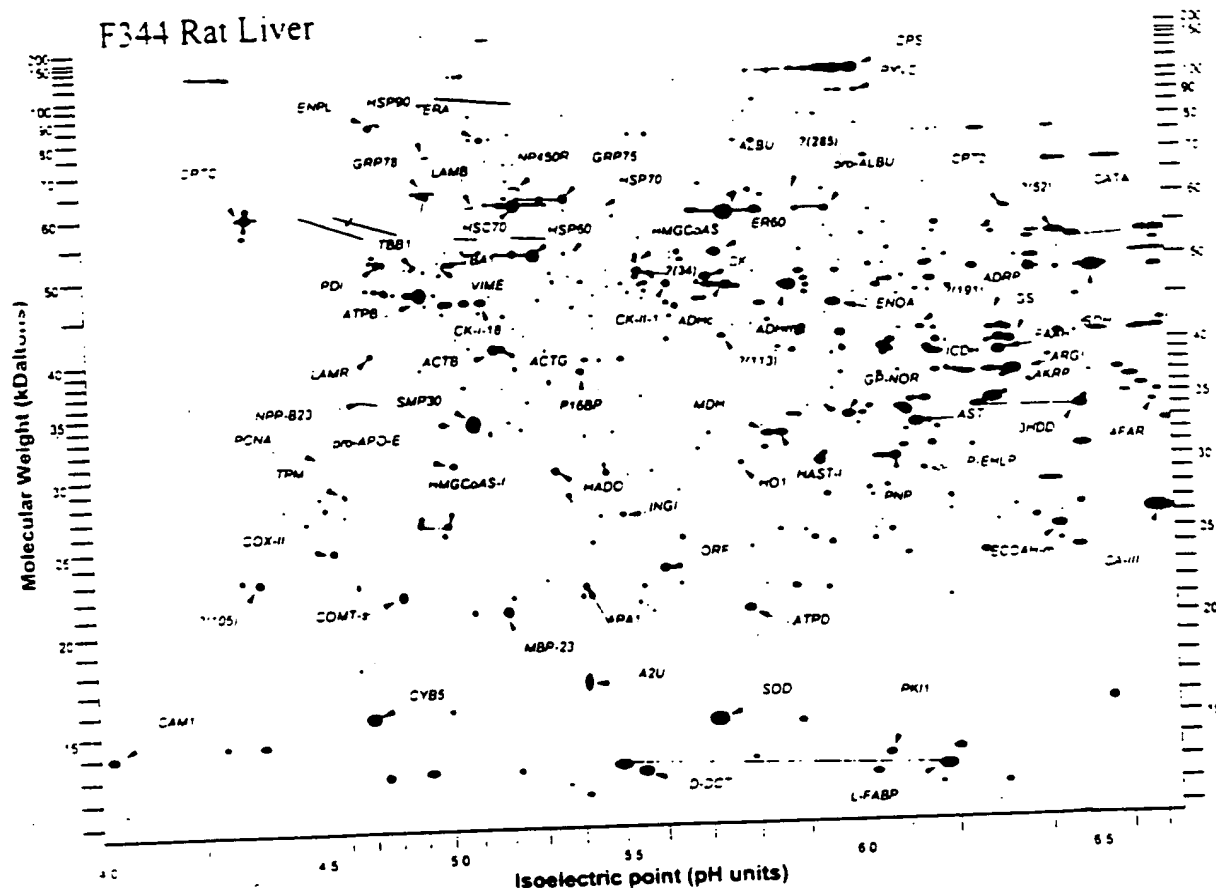


Fig. 2. Computerized representation of a Coomassie Blue stained two-dimensional gel electrophoresis pattern of Fischer F344 rat liver homogenate.

quantitative expression data has been collected, is to visualize complex patterns of gene expression changes, to detect pathways and sets of genes tightly correlated with treatment efficacy and toxicity, and to compare the effects of different sets of treatment (Anderson et al., 1996). As the drug effect database is growing, one may detect similarities and differences between the molecular fingerprints produced by various drugs, information that may be crucial to make a decision whether to refocus or extend the therapeutic spectrum of a drug candidate.

### 5. Comparison of global mRNA and protein expression profiling

There are several synergies and overlaps of data obtained by mRNA and protein expression analysis. Low abundant transcripts may not be easily quantified at the protein level using standard two-dimensional gel electrophoresis analysis and their detection may require prefractionation of samples. The expression of such genes may be preferably quantified at the mRNA level using techniques allowing PCR-mediated target amplifi-

cation. Tissue biopsy samples typically yield good quality of both mRNA and proteins; however, the quality of mRNA isolated from body fluids is often poor due to the faster degradation of mRNA when compared with proteins. RNA samples from body fluids such as serum or urine are often not very 'meaningful', and secreted proteins are likely more reliable surrogate markers for treatment efficacy and safety. Detection of post-translational modifications, events often related to function or nonfunction of a protein, is restricted to protein expression analysis and rarely can be predicted by mRNA profiling. Information on subcellular localization and translocation of proteins has to be acquired at the level of the protein in combination with sample prefractionation procedures. The growing evidence of a poor correlation between mRNA and protein abundance (Anderson and Seilhamer, 1997) further suggests that the two approaches, mRNA and protein profiling, are complementary and should be applied in parallel.

## 6. Expression profiling and drug development

Understanding the mechanisms of action and toxicity, and being able to monitor treatment efficacy and safety during trials is crucial for the successful development of a drug. Mechanistic insights are essential for the interpretation of drug effects and enhance the chances of recognizing potential species specificities contributing to an improved risk profile in humans (Richardson et al., 1993; Steiner et al., 1996b; Aicher et al., 1998). The value of expression profiling further increases when links between treatment-induced expression profiles and specific pharmacological and toxic endpoints are established (Anderson et al., 1991, 1995, 1996; Steiner et al., 1996a). Changes in gene expression are known to precede the manifestation of morphological alterations, giving expression profiling a great potential for early compound screening, enabling one to select drug candidates with wide therapeutic windows reflected by molecular fingerprints indicative of high pharmacological potency and low toxicity (Arce et al., 1998). In later phases of drug devel-

opment, surrogate markers of treatment efficacy and toxicity can be applied to optimize the monitoring of pre-clinical and clinical studies (Doherty et al., 1998).

## 7. Perspectives

The basic methodology of safety evaluation has changed little during the past decades. Toxicity in laboratory animals has been evaluated primarily by using hematological, clinical chemistry and histological parameters as indicators of organ damage. The rapid progress in genomics and proteomics technologies creates a unique opportunity to dramatically improve the predictive power of safety assessment and to accelerate the drug development process. Application of gene and protein expression profiling promises to improve lead selection, resulting in the development of drug candidates with higher efficacy and lower toxicity. The identification of biologically relevant surrogate markers correlated with treatment efficacy and safety bears a great potential to optimize the monitoring of pre-clinical and clinical trials.

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likely, its power can be attributed to its incorporation of more information than any other measure; it takes account of the full substitution and gap data (like raw scores) but also has details about the sequence lengths and composition and is scaled appropriately.

We find that statistical scores are not only powerful, but also easy to interpret. SSEARCH and FASTA show close agreement between statistical scores and actual number of errors per query (Fig. 4). The expectation value score gives a good, slightly conservative estimate of the chances of the two sequences being found at random in a given query. Thus, an E-value of 0.01 indicates that roughly one pair of nonhomologs of this similarity should be found in every 100 different queries. Neither raw scores nor percentage identity can be interpreted in this way, and these results validate the suitability of the extreme value distribution for describing the scores from a database search.

The P-values from BLAST also should be directly interpretable but were found to overstate significance by more than two orders of magnitude for 1% EPQ for this database. Nonetheless, these results strongly suggest that the analytic theory is fundamentally appropriate. WU-BLAST2 scores were more reliable than those from BLAST, but also exaggerate expected confidence by more than an order of magnitude at 1% EPQ.

**Overall Detection of Homologs and Comparison of Algorithms.** The results in Fig. 5A and Table 1 show that pairwise sequence comparison is capable of identifying only a small fraction of the homologous pairs of sequences in PDB40D-B. Even SSEARCH with E-values, the best protocol tested, could find only 18% of all relationships at a 1% EPQ. BLAST, which identifies 15%, was the worst performer, whereas FASTA ktup = 1 is nearly as effective as SSEARCH. FASTA ktup = 2 and WU-BLAST2 are intermediate in their ability to detect homologs. Comparison of different algorithms indicates that those capable of identifying more homologs are generally slower. SSEARCH is 25 times slower than BLAST and 6.5 times slower than FASTA ktup = 1. WU-BLAST2 is slightly faster than FASTA ktup = 2, but the latter has more interpretable scores.

In PDB90D-B, where there are many close relationships, the best method can identify only 38% of structurally known homologs (Fig. 5B). The method which finds that many relationships is WU-BLAST2. Consequently, we infer that the differences between FASTA ktup = 1, SSEARCH, and WU-BLAST2 programs are unlikely to be significant when compared with variation in database composition and scoring reliability.

Fig. 6 helps to explain why most distant homologs cannot be found by sequence comparison: a great many such relationships have no more sequence identity than would be expected by chance. SSEARCH with E-values can recognize >90% of the homologous pairs with 30–40% identity. In this region, there are 30 pairs of homologous proteins that do not have significant E-values, but 26 of these involve sequences with <50 residues. Of sequences having 25–30% identity, 75% are identified by SSEARCH E-values. However, although the number of homologs grows at lower levels of identity, the detection falls off sharply: only 40% of homologs with 20–25% identity

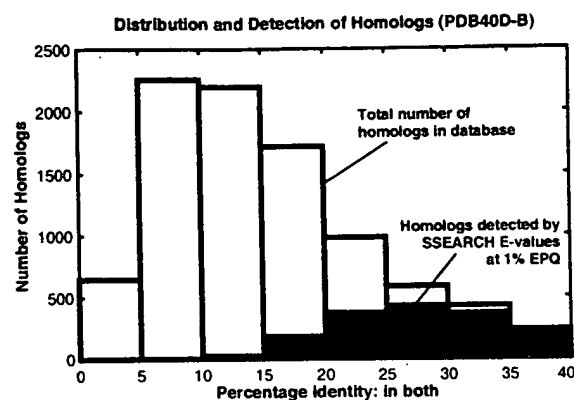


FIG. 6. Distribution and detection of homologs in PDB40D-B. Bars show the distribution of homologous pairs PDB40D-B according to their identity (using the measure of identity in both). Filled regions indicate the number of these pairs found by the best database searching method (SSEARCH with E-values) at 1% EPQ. The PDB40D-B database contains proteins with <40% identity, and as shown on this graph, most structurally identified homologs in the database have diverged extremely far in sequence and have <20% identity. Note that the alignments may be inaccurate, especially at low levels of identity. Filled regions show that SSEARCH can identify most relationships that have 25% or more identity, but its detection wanes sharply below 25%. Consequently, the great sequence divergence of most structurally identified evolutionary relationships effectively defeats the ability of pairwise sequence comparison to detect them.

are detected and only 10% of those with 15–20% can be found. These results show that statistical scores can find related proteins whose identity is remarkably low; however, the power of the method is restricted by the great divergence of many protein sequences.

After completion of this work, a new version of pairwise BLAST was released: BLASTGP (37). It supports gapped alignments, like WU-BLAST2, and dispenses with sum statistics. Our initial tests on BLASTGP using default parameters show that its E-values are reliable and that its overall detection of homologs was substantially better than that of ungapped BLAST, but not quite equal to that of WU-BLAST2.

## CONCLUSION

The general consensus amongst experts (see refs. 7, 24, 25, 27 and references therein) suggests that the most effective sequence searches are made by (i) using a large current database in which the protein sequences have been complexity masked and (ii) using statistical scores to interpret the results. Our experiments fully support this view.

Our results also suggest two further points. First, the E-values reported by FASTA and SSEARCH give fairly accurate estimates of the significance of each match, but the P-values provided by BLAST and WU-BLAST2 underestimate the true

Table 1. Summary of sequence comparison methods with PDB40D-B

Method	Relative Time*	1% EPQ Cutoff	Coverage at 1% EPQ
SSEARCH % identity: within alignment	25.5	>70%	<0.1
SSEARCH % identity: within both	25.5	34%	3.0
SSEARCH % identity: HSSP-scaled	25.5	35% (HSSP + 9.8)	4.0
SSEARCH Smith–Waterman raw scores	25.5	142	10.5
SSEARCH E-values	25.5	0.03	18.4
FASTA ktup = 1 E-values	3.9	0.03	17.9
FASTA ktup = 2 E-values	1.4	0.03	16.7
WU-BLAST2 P-values	1.1	0.003	17.5
BLAST P-values	1.0	0.00016	14.8

\*Times are from large database searches with genome proteins.

extent of errors. Second, SSEARCH, WU-BLAST2, and FASTA ktup = 1 perform best, though BLAST and FASTA ktup = 2 detect most of the relationships found by the best procedures and are appropriate for rapid initial searches.

The homologous proteins that are found by sequence comparison can be distinguished with high reliability from the huge number of unrelated pairs. However, even the best database searching procedures tested fail to find the large majority of distant evolutionary relationships at an acceptable error rate. Thus, if the procedures assessed here fail to find a reliable match, it does not imply that the sequence is unique; rather, it indicates that any relatives it might have are distant ones.\*\*

\*\*Additional and updated information about this work, including supplementary figures, may be found at <http://sss.stanford.edu/sss/>.

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**Subject: RE: [Fwd: Toxicology Chip]**

**Date: Mon, 3 Jul 2000 08:09:45 -0400**

**From: "Afshari, Cynthia" <afshari@niehs.nih.gov>**

**To: "Diana Hamlet-Cox" <dianahc@incyte.com>**

You can see the list of clones that we have on our 12K chip at  
<http://manuel.niehs.nih.gov/maps/guest/clonesrch.cfm>

We selected a subset of genes (2000K) that we believed critical to tox response and basic cellular processes and added a set of clones and ESTs to this. We have included a set of control genes (80+) that were selected by the NHGRI because they did not change across a large set of array experiments. However, we have found that some of these genes change significantly after tox treatments and are in the process of looking at the variation of each of these 80+ genes across our experiments.

Our chips are constantly changing and being updated and we hope that our data will lead us to what the toxchip should really be.  
I hope this answers your question.

Cindy Afshari

> -----

> From: Diana Hamlet-Cox

> Sent: Monday, June 26, 2000 8:52 PM

> To: afshari@niehs.nih.gov

> Subject: [Fwd: Toxicology Chip]

>

> Dear Dr. Afshari,

>

> Since I have not yet had a response from Bill Grigg, perhaps he was not  
> the right person to contact.

>

> Can you help me in this matter? I don't need to know the sequences,  
> necessarily, but I would like very much to know what types of sequences  
> are being used, e.g., GPCRs (more specific?), ion channels, etc.

>

> Diana Hamlet-Cox

>

> ----- Original Message -----

> Subject: Toxicology Chip

> Date: Mon, 19 Jun 2000 18:31:48 -0700

> From: Diana Hamlet-Cox <dianahc@incyte.com>

> Organization: Incyte Pharmaceuticals

> To: grigg@niehs.nih.gov

>

> Dear Colleague:

>

> I am doing literature research on the use of expressed genes as  
> pharmacotoxicology markers, and found the Press Release dated February  
> 29, 2000 regarding the work of the NIEHS in this area. I would like to  
> know if there is a resource I can access (or you could provide?) that  
> would give me a list of the 12,000 genes that are on your Human ToxChip  
> Microarray. In particular, I am interested in the criteria used to  
> select sequences for the ToxChip, including any control sequences  
> included in the microarray.

>

> Thank you for your assistance in this request.

>

> Diana Hamlet-Cox, Ph.D.

> Incyte Genomics, Inc.

>

> --

>

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